Behavioural and olfactory responses of pest insects to endophyte-infected (*Epichloë festucae* variant *lolii*) perennial ryegrass (*Lolium perenne*)

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Asexual fungal endophytes (*Epichloë*) colonise agricultural grasses (Poaceae) in an interaction which provides host plants with protection against herbivorous insects. Despite 40 years of research there is still much we do not know about these complex interactions. A major gap in our knowledge is an understanding of the mechanisms involved in perception of endophyte by host-searching insects. It has, perhaps, been assumed that perception of endophyte is mediated by ingestion of endophyte-derived alkaloids, resulting in a malaise and an avoidance response. Although a post-ingestional malaise is one theory it is also feasible that insects detect endophyte via sensory perception before ingesting plant material. Sensory perception involves olfactory and/or contact (gustatory) chemoreception and is often referred to as an insect's 'sense of smell and taste'. I explored the mechanisms involved in insect perception of the endophyte, *Epichloë festucae* variant *lolii* (Latch, M. J. Chr. & Samuels, Hypocreales: Clavicipitaceae), in perennial ryegrass (*Lolium perenne* Linnaeus, Poales: Poaceae) hosts.

When presented with a choice a root aphid, *Aploneura lentisci*, was not deterred from endophyte-infected (AR37 strain) ryegrass in a host-preference assay and thus appeared to be unable to initially (24 h) perceive endophyte, demonstrating that negative effects of endophyte are not always associated with initial perception and avoidance responses. Further olfactometer experiments demonstrated that under the experimental conditions I used, apterous nymphs were unable to utilise olfaction during host-searching, suggesting that this morph cannot perceive host plants before contact is made with the plant. In contrast to this, olfaction was an important sensory mechanism for Argentine stem weevil adults (ASW, *Listronotus bonariensis*) which employed olfaction to locate host plants and distinguish between undamaged and herbivore damaged hosts. Olfaction is mediated by plant volatiles and both endophyte and herbivory were shown to alter the blend emitted by perennial ryegrass in this study.

ASW perceive certain endophyte strains and I propose a role of contact chemoreception in perception. Four lines of evidence were presented that support this theory. In the olfactometer bioassays; (1) there was no evidence that ASW avoided the odour blend released by endophyte-infected ryegrass before (AR1 or common-toxic) or after (AR1) plants had been damaged by conspecific insects. In the whole plant choice experiment; (2) there was no evidence that ASW utilised precontact cues (olfaction and vision) to orient away from endophyte-infected (AR1) plants from the outset; (3) ASW showed a strong aversion to endophyte-infected plants with just eight of 45 weevils observed feeding on AR1-infected plants and only one weevil feeding on both hosts during the observational period. In comparison, 32 weevils were observed feeding on endophyte-free plants (4 did not feed); (4) grooming of chemosensory appendages was only observed in weevils enclosed with endophyte-infected plants (AR1 or common-toxic) in the no-choice experiment.

This thesis has established a framework, based on investigations of pre- and post- contact behaviour, for investigating mechanisms of insect perception of endophyte and this can be utilised in future studies. Furthermore, this study has identified effects of endophyte on behaviours that have not previously been reported in the endophyte literature and this has provided an exciting area for future research.

Keywords: Endophyte, *Epichloë festucae* variant *lolii*, perennial ryegrass, *Lolium perenne*, Argentine stem weevil, *Listronotus bonariensis*, root aphid, *Aploneura lentisci*, chemoreception, olfaction, volatile organic compounds, herbivore-induced plant volatiles, gustation, contact chemoreception, post-ingestion, alkaloids, host-selection, host-searching, perception, behaviour.

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This journey started a decade ago when I left high school as an over-eager, super-optimistic, 17-year-old ready to take on the world and pursue a career in science. Ten years later, I have a few fancy pieces of paper and I know a lot about insects and grass making for great work stories.

Boom

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Chapter 1

Introduction

Synthetic pesticides are used to control invertebrate pests in agricultural and horticultural ecosystems worldwide. Insecticide applications kill or deter insects, which often results in significant increases in crop yield and quality. However, there is a growing awareness of the impacts these chemicals can have on the environment and welfare of humans and other mammals (Pimentel *et al.*, 1992; Tilman *et al.*, 2002). This has resulted in increased demand for sustainable alternatives that do not reduce plant quality or productivity and are effective under a diverse range of environmental conditions. Naturally occurring biological controls, such as microbial endophytes, have the potential to be sustainable alternatives.

The term endophyte in its simplest form originates from the Greek words 'endon' and 'phyton' meaning an organism living within a plant (De Bary, 1866; Wennström, 1994; Wilson, 1995). Endophytic organisms are commonly fungi or bacteria, but the term can also encompass viruses as well as other plants (e.g. mistletoe) that live within a host for all, or at least part, of their lifecycle (Calvin, 1967; Wilson, 1995; Chanway, 1996). Endophytes are incredibly diverse and can be found within the leaves, stems, flowers, fruits and seeds of host plants, where they form associations that can range from antagonistic to mutualistic (Schulz & Boyle, 2006). Some of these associations provide host plants with protection against insect pests and plant diseases and it is these interactions that are now exploited as novel biological controls (Glare *et al.*, 2012; Johnson *et al.*, 2013).

Examples of endophytes used as biological controls are entomopathogenic fungi, such as the ubiquitous *Beauveria bassiana*, which forms mutualistic associations with several important crops including banana, coffee, cotton, opium poppy and cocoa (Posada & Vega, 2005, 2006; Quesada-Moraga *et al.*, 2006; Lopez *et al.*, 2014). The potential of this fungus as a biological control has been demonstrated in several crop species, including banana (*Musa* spp.) where Akello *et al.* (2008) showed that endophyte infection reduced the survival of banana weevil larvae (*Cosmopolites sordidus*). In a similar way, Bing and Lewis (1991) demonstrated a reduction in tunnelling by the European corn borer (*Ostrinia nubilalis*, Lepidoptera: Crambidae) in *B. bassiana* infected maize leaves (*Zea mays*). Another example is the *Fusarium* species which has been shown to reduce the severity of root galling caused by a root knot nematode (*Meloidogyne graminicola*, Tylenchida: Meloidogynidae) in rice (Le *et al.*, 2009), root penetration of a burrowing nematode (*Radopholus similis*, Tylenchida: Pratylenchidae) in banana (Vu *et al.*, 2006) as well as the number of galls produced by *Meloidogyne incognita* (Tylenchida: Meloidogynidae) in tomato plants (Hallmann & Sikora, 1994b, 1994a).

Endophytes of the Genus *Epichloë* that occur under both sexual and asexual morphs are, arguably, the most well-documented to date (Clay, 1988; Johnson *et al.*, 2013). Sexual morphs are horizontally (contagiously) transmitted and can behave antagonistically towards their hosts as they form stromata on immature grass inflorescences, supressing the development of these structures (termed "choke disease") (White, 1988; Schardl *et al.*, 2004). Asexual morphs on the other hand, are vertically transmitted within the seed of their host and form mutualistic associations with agricultural grasses (Figure 1.1). These obligate biotrophs have been the subject of extensive study over the past four decades and several strains (genotypes) have been successfully commercialised (Popay & Hume, 2011; Caradus *et al.*, 2013a; Johnson *et al.*, 2013; Pennell & Rolston, 2013; Young *et al.*, 2013; Malinowski & Belesky, 2019). Throughout this thesis the term 'endophyte' will be used to refer specifically to asexual morphs of the genus *Epichloë*.

1.1 Asexual *Epichloë* endophytes

Asexual fungal endophytes (formally *Neotyphodium* spp. (Leuchtmann *et al.*, 2014) and *Acremonium* spp. (Glenn *et al.*, 1996)) do not have an external form and grow as unbranched hyphae through the intercellular spaces of their host plant (Christensen *et al.*, 2002). Hyphae asymptomatically colonise the aboveground tissues of temperate grasses in a relationship which is described as defensive mutualistic (Clay, 1988; Saikkonen *et al.*, 2010). In this interaction the endophyte gains nutrients, shelter and a means of transmission within the seed of the host plant (Philipson & Christey, 1986; Clay & Schardl, 2002). In return, the endophyte protects its host from biotic and abiotic stress, increasing the plant's resilience to insects, nematodes, pathogens and drought (Bacetty *et al.*, 2009; Nagabhyru *et al.*, 2013; Pańka *et al.*, 2013b; Hennessy *et al.*, 2016; Popay & Cox, 2016; Malinowski & Belesky, 2019). Protection from insects has primarily been attributed to the production of secondary metabolites which can have anti-feedant and/or toxic effects on pest insects (Popay *et al.*, 1990; Popay & Lane, 2000; Hennessy *et al.*, 2016).

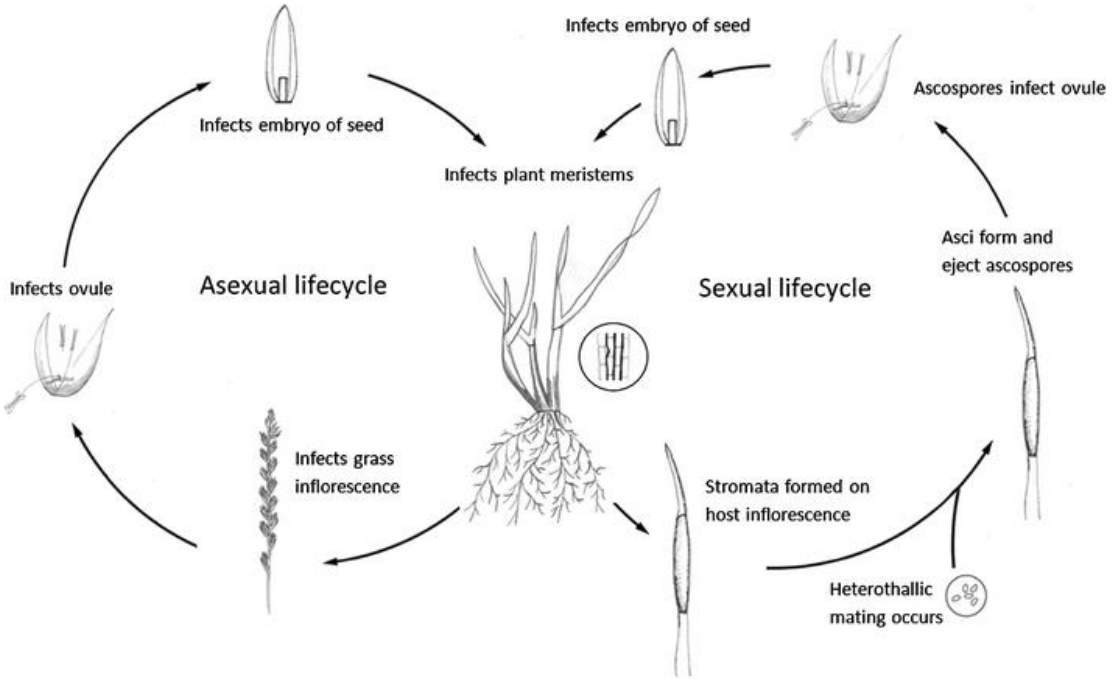


Figure 1.1: Asexual and sexual lifecycles of fungal endophytes from the genus *Epichloë* within a grass host (Poaceae). Reproduced with permission from Johnson *et al.* (2013).

Intensive pastoral endophyte research began in the early 1980s following simultaneous discoveries in New Zealand and the United States of America that fungal endophytes in grasses can be associated with livestock toxicosis (Bacon *et al.*, 1977; Fletcher & Harvey, 1981; Gallagher *et al.*, 1981; Gallagher *et al.*, 1984; Yates *et al.*, 1985). In New Zealand, livestock feeding on the predominant agricultural grass species, perennial ryegrass (*Lolium perenne* Linnaeus, Poales: Poaceae), were prone to developing ryegrass staggers and heat stress under certain grazing conditions (Cunningham & Hartley, 1959; Fletcher & Harvey, 1981; Fletcher, 1993). Ryegrass staggers is a neurological impairment and symptoms typically involve spasms and stiffness of the limbs after a period of exercise, although in more severe cases an animal may experience tetanic spasms or remain continuously prostrate (Cunningham & Hartley, 1959). Animals suffering from heat stress can have high respiration rates and may pant and drool which can culminate in a loss of condition and production (Easton *et al.*, 1996). Fletcher and Harvey (1981) were the first to suggest an association between ryegrass staggers and the fungal endophyte (known variously as the common-toxic endophyte (CT), wild-type or standard endophyte) found within perennial ryegrass in New Zealand. The mycotoxins responsible for toxicosis were then identified as lolitrem B (ryegrass staggers) and later ergovaline (heat stress) (Gallagher *et al.*, 1981; Gallagher *et al.*, 1982; Gallagher *et al.*, 1984; Easton *et al.*, 1996). A logical solution was to remove the endophyte from perennial ryegrass, but this was not a viable option as endophyte-free perennial ryegrass was shown to be highly susceptible to Argentine stem weevil (*Listronotus bonariensis* (Kuschel, 1955), Coleoptera: Curculionidae) feeding damage (Prestidge *et al.*, 1982;

Mortimer & di Menna, 1983). Rowan and Gaynor (1986) were subsequently able to identify an alkaloid, peramine, in endophyte-infected ryegrass that conferred resistance to ASW.

As a result of these discoveries, endophyte research began to focus on identifying alternative endophyte strains that provided the host plant with protection against phytophagous insects but had no, or limited, animal health effects. Initial selection criteria for these strains were that they should contain peramine and not the mycotoxin lolitrem B. Hundreds of perennial ryegrass plants from around New Zealand were initially sampled, but all were found to produce lolitrem B (Tapper & Latch, 1999). As a result, the search was extended to include wild perennial ryegrass in Europe as well as seed from the United States Department of Agriculture forage grass seed collection (Tapper & Latch, 1999). A higher degree of chemical diversity was found among these plants and several endophyte-infected grasses that met selection criteria were identified. The fungal endophytes colonising these plants were cultured and inoculated into New Zealand ryegrass cultivars leading to the release of 'Endosafe' – the first 'selected' endophyte strain. Certain ryegrass cultivar combinations with 'Endosafe' were later withdrawn from the market as ergovaline concentrations were found to accumulate at certain stages of development such as flowering, causing heat stress and poor liveweight gains in livestock (Tapper & Latch, 1999). As a result, the screening process was expanded to include ergovaline. Since then a number of different strains have been identified and the 'selected' (or 'novel') endophyte strains commercially available now include AR1, AR37, NEA2, Endosafe, Happe, Edge and Avanex® in ryegrass species, MaxQ® (also known as MaxP®) and Protek® in tall fescue (*Festuca arundinacea*) as well as the GrubOUT® U2 endophyte in meadow fescue (*Festuca pratensis*) and its hybrids (Johnson *et al.*, 2013; Young *et al.*, 2013). In New Zealand in particular this technology has had a rapid uptake from farmers (Caradus *et al.*, 2013b) with 'selected' endophyte strains contributing at least $200 million dollars (NZD) to the economy annually (Johnson *et al.*, 2013; Ferguson *et al.*, 2018).

'Selected' endophytes are sold to farmers within the seed of the host grass and licensing agreements for the AR1 and AR37 strains, which dominate the ryegrass proprietary seed market in New Zealand, dictate that >70% of the seed in a given seed line must contain viable endophyte (Hume & Barker, 2005; Hume *et al.*, 2013). In reality endophyte viability within the seed is influenced by storage time, temperature and humidity (Rolston *et al.*, 1986; Hume *et al.*, 2013) and suboptimum storage conditions will result in lower infection rates when these seeds are sown in the field. It is also not mandatory to meet this high level of infection and uncertified seed can be planted. These are key aspects to consider when investigating these complex interactions in New Zealand's intensive pastoral ecosystems.

AR1 was commercialised in 2001 and was rapidly adopted by farmers as it provided protection against a key pest, Argentine stem weevil, without producing the mycotoxins lolitrem B and ergovaline which

affect grazing livestock (Fletcher, 1999; Popay *et al.*, 1999). However, this strain did not provide strong protection against African black beetle (*Heteronychus arator*, Coleoptera: Scarabaeidae), which are a major pasture pest in the warmer regions of the country (Popay & Baltus, 2001). The AR37 endophyte was commercialised in 2007, despite producing the indole diterpene, epoxy-janthitrem (Tapper & Lane, 2004; Finch *et al.*, 2010), which can cause mild ryegrass staggers in sheep under certain grazing conditions (Thom *et al.*, 2007; Fletcher & Sutherland, 2009). AR37 quickly dominated the market as it provides superior control over a wide range of pests compared to AR1, including Argentine stem weevil larvae, African black beetle adults, porina larvae (*Wiseana* spp., Lepidoptera: Hepialidae) and a root aphid (*Aploneura lentisci* Passerini, 1856, Hemiptera: Aphididae) (Popay & Wyatt, 1995; Jensen & Popay, 2004; Hume *et al.*, 2007; Popay & Gerard, 2007; Popay & Thom, 2009; Popay *et al.*, 2012). The AR1 and CT strains are more closely related to each other than they are to the AR37 endophyte strain and share a similar chemical profile (Figure 1.2).

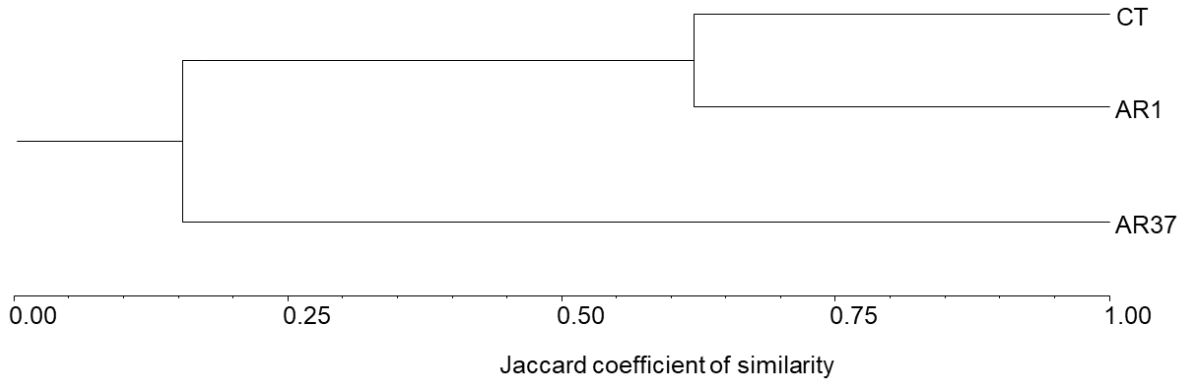


Figure 1.2: Dendrogram depicting the genetic relatedness (estimate from 24 simple sequence repeat DNA markers) of the three predominant endophyte strains in New Zealand's intensive pastoral ecosystems (M. J. Faville, personal communication).

1.1.1 Endophyte-derived alkaloids

Alkaloids, organic compounds that contain at least one nitrogen atom, are a large class of naturally occurring compounds that includes the well-known pharmaceuticals morphine, codeine, atropine, ephedrine, caffeine and nicotine. Alkaloids are secondary plant compounds and are known to be involved in defence against predators and pathogens (Oliva *et al.*, 2003; Chowański *et al.*, 2016). The anti-insect properties of *Epichloë* infected ryegrass are commonly attributed to endophyte-derived alkaloids (Table 1.1). Among the well characterised alkaloids are the indole diterpenes, lolitrem B and epoxy-janthitrems, ergot alkaloids, lolines and the pyrrolizidine alkaloid peramine (Gallagher *et al.*, 1984; Rowan & Gaynor, 1986; Munday-Finch *et al.*, 1995; Munday-Finch *et al.*, 1996; Finch *et al.*, 2010). Concentrations of these compounds *in planta* can be affected by host plant species and ploidy as well

as abiotic growth conditions such as temperature, water availability and nitrogen supply (Lane *et al.*, 1997; Salminen *et al.*, 2005; Rasmussen *et al.*, 2007; Hennessy *et al.*, 2016). Alkaloid concentrations within different plant parts varies between alkaloids. Epoxy-janthitrem, lolitrem B and ergovaline are concentrated in the pseudostem, where endophyte mycelium is concentrated, due to the lipophilic nature of these compounds which results in low in planta mobility (di Menna *et al.*, 1992; Ball *et al.*, 1997b; Munday-Finch & Garthwaite, 1999; Spiering *et al.*, 2002; Hennessy *et al.*, 2016). Conversely, peramine has a uniform distribution within the herbage of the plant as this compound is hydrophilic and therefore highly translocatable (Ball *et al.*, 1997a; Spiering *et al.*, 2002). Endophyte hyphae do not colonise the roots but the properties of some alkaloids, such as lolines, allow them to be transported into the root systems of certain hosts. Lolines can be found in the roots of tall fescue infected with the endophyte *Epichloë coenophiala* (formerly *Neotyphodium coenophiala*) and meadow fescue infected with *Epichloë uncinata* (formerly *Neotyphodium uncinatum*) and can provide the plant with some protection against root-feeding insects (Wilkinson *et al.*, 2000; Barker *et al.*, 2015).

Bioactivity of alkaloids can be assessed in semi-synthetic (or artificial) diet experiments (Popay *et al.*, 1990; Rowan *et al.*, 1990; Popay & Lane, 2000; Jensen *et al.*, 2009; Popay *et al.*, 2009; Hennessy, 2015; Hennessy *et al.*, 2016). In these experiments, pure compounds or semi-pure extracts are incorporated into diet and consumption, growth and survival of the insect is monitored over a period of time. Results from these experiments have demonstrated that alkaloids can have anti-feedant effects on herbivorous pest insects. Epoxy-janthitrems (a group of 5 compounds), for example, were shown to have a strong anti-feedant effect on porina larvae, which are a major pasture pest in New Zealand. In a no-choice bioassay, larvae presented with a diet containing semi-pure epoxy-janthitrem I, extracted from AR37-infected ryegrass seed, fed significantly less and gained significantly less weight than larvae fed an equivalent control diet (Hennessy, 2015). This anti-feedant effect was demonstrated at low, medium and high concentrations (1, 2.5 and 5 µg/g) (Hennessy, 2015). In addition to the known alkaloids, it is reasonable to predict that there will be other, as yet unknown, compounds which may be involved in mediating deterrent effects as some chemistry of the interactions have yet to be fully resolved. These compounds may be alkaloids or they may belong to other chemical classes.

Table 1.1: Alkaloid profile, insects affected and animal health disorders associated with the common ryegrass (*Lolium perenne*) endophyte strains in New Zealand.

Endophyte strain	Known alkaloids	Affected insects[1]	Animal health disorders
Common-toxic (wild type or standard endophyte) (*Epichloë festucae* variant *lolii*)	Lolitrem B, ergovaline and peramine	Argentine stem weevil adult and larvae (*Listronotus bonariensis*) (Barker *et al.*, 1984a; Barker *et al.*, 1984b) African black beetle adult (*Heteronychus arator*) (Ball & Prestidge, 1992) Porina larvae (*Wiseana* spp.) (Jensen & Popay, 2004) Pasture mealybug (*Balanococcus poae*) (Pennell et al., 2005)	Ryegrass staggers and heat stress (Fletcher & Harvey, 1981; Gallagher et al., 1981; Gallagher et al., 1984)
AR1 (*Epichloë festucae* variant *lolii*)	Peramine	Argentine stem weevil adult and larvae (*Listronotus bonariensis*) (Popay *et al.*, 1990; Popay *et al.*, 1999) Pasture mealybug (*Balanococcus poae*) (Pennell et al., 2005)	No negative effects (Fletcher, 1999)
AR37 (*Epichloë festucae* variant *lolii*)	Epoxy-janthitrems	Argentine stem weevil larvae (*Listronotus bonariensis*) (Popay & Wyatt, 1995) Root aphid (*Aploneura lentisci*) (Popay & Cox, 2016) African black beetle adult (*Heteronychus arator*) (Ball et al., 1994) Porina larvae (*Wiseana* spp.) (Jensen & Popay, 2004; Hennessy et al., 2016) Pasture mealybug (*Balanococcus poae*) (Pennell et al., 2005)	Ryegrass staggers in sheep but less severe than common-toxic. No heat stress (Thom *et al.*, 2007; Fletcher & Sutherland, 2009)

[1] Table includes 5 of the 6 major pasture pests as none of the strains provide protection against grass grub larvae (*Costelytra giveni*). AR1 can provide some protection against African black beetle adults but ryegrass remains suseptible to damage (Popay & Baltus, 2001). The common-toxic strain can negatively impact root aphid populations but negative effects are not consistent (Popay *et al.*, 2004; Popay & Gerard, 2007).

1.2 Pastoral ecosystems in New Zealand

Following European settlement in New Zealand in the 18th century, vast areas of indigenous vegetation were felled and cleared for farming. Millions of hectares are currently dedicated to producing meat and milk products from farming dairy cows, sheep and beef cattle. Farmland in New Zealand is characterised by its low species diversity and the dominance of ryegrass (*Lolium perenne* and *Lolium multiflorum*) and white clover swards (*Trifolium repens*) (Charlton & Stewart, 1999). With little supplementary feeding, livestock are reliant on high-quality ryegrass, the production of which is strongly influenced by invertebrate pests (Prestidge *et al.*, 1991; Zydenbos *et al.*, 2011; Ferguson *et al.*, 2018). Chewing, rasping and sucking insects feed on the herbage and roots of these plants causing damage ranging from leaf scarring to tiller death. Recent estimates have placed the cost of damage from the major pests to be as high as $2.3 billion NZD in an 'average' year (Ferguson *et al.*, 2018). Costs are likely to be higher in pest outbreak years or following climatic events such as drought which often compound losses (Ferguson *et al.*, 2018). Therefore, substantial gains are to be made with continual improvement of pest management strategies.

Farmland ecosystems in New Zealand provide a unique study system as the scarcity of natural predators has allowed introduced pests as well as some endemic species, that have adapted well to pastoral species, to flourish. In this thesis I have focused on two major pests: the below ground root feeding aphid, *Aploneura lentisci*, and the foliage feeding Argentine stem weevil adult, *Listronotus bonariensis*.

1.2.1 *Aploneura lentisci* (Passerini, 1856, Hemiptera: Aphididae)

In the Mediterranean, this aphid's native range, *A. lentisci* has a complex two-year holocyclic life cycle (can reproduce parthenogenetically and sexually) alternating between galls on the leaves of its primary host, *Pistacia lentiscus* (Anacardiaceae) and the roots of its secondary host, Poaceae (Wool & Manheim, 1986). Apterous (wingless) individuals are produced throughout much of the life cycle and alatae (winged) morphs are formed in the last generation for migration between primary and secondary hosts (Wool & Manheim, 1986; Wool *et al.*, 1994; Wool, 1995).

In New Zealand *A. lentiscus* (known by its common name root aphid) is a chronic pest of perennial ryegrass and tall fescue (Popay & Cox, 2016). The primary host, *P. lentisci*, is not found in New Zealand and apterous parthenogenetic females occur year-round on the roots of Poaceae (Popay & Cox, 2016). Apterous nymphs are highly mobile through the soil and on the surface and have been recorded on plant herbage (Rasmussen *et al.*, 2008). Mature apterous aphids are largely sedentary and remain attached to the root system protected by a thick coating of white flocculant wax. Aphid distribution is highly aggregated but occurs throughout the root system, from the crown to at least 100 mm deep

(Pennell *et al.*, 2005). Alatae *A. lentisci* were occasionally found in grease traps established at two sites in Canterbury, New Zealand between 1962 and 1966 (Lowe, 1968) and they were found in a colony that was maintained in a climate chamber (Müller, 2019). However, it is not clear whether these alatae were sexupare (aphids produced for dispersal to primary hosts where they give birth to sexuales) or alatae virginoparae (produced by parthenogenesis). Blackman and Eastop (2000) reported that flights of alatae in New Zealand in the late summer were largely made up of sexupare. Whether alatae virginoparae are commonly produced in pastoral farmland in New Zealand is not known (Müller, 2019). However, since alatae have not been observed when sampling below ground populations in the field, Popay and Cox (2016) hypothesised that apterous nymphs may primarily be responsible for dispersal of the clone between secondary host plants in New Zealand.

Due to the inherent difficulties of studying below ground insects, little is known about the biology and ecology of this pest in New Zealand and its impacts on plant health. As a result, a recent review of New Zealand's pasture pests by Ferguson *et al.* (2018) could not assess the pest status and economic importance of this aphid. However, the large population sizes found on some plants suggests that this aphid can have severe impacts especially when combined with additional stressors such as drought, heavy grazing or other insect attack (Pennell *et al.*, 2005; Popay & Gerard, 2007; Salmon *et al.*, 2008).

Although transitory, the CT endophyte can have some effect on *A. lentisci*, but AR1 has no negative effect (Popay *et al.*, 2004; Hume *et al.*, 2007; Popay & Gerard, 2007; Popay & Thom, 2009). In contrast, the AR37 endophyte strain has a potent effect on *A. lentisci* which results in low aphid populations and increased growth of plants containing this endophyte (Pennell *et al.*, 2005; Popay & Gerard, 2007; Popay & Cox, 2016). The epoxy-janthitrem alkaloids are unique to the AR37 endophyte strain but it is not clear whether these compounds are responsible for bioactivity against this aphid as, due to their lipophilic nature, they would not be easily translocated into the root system.

1.2.2 Argentine stem weevil (*Listronotus bonariensis* (Kuschel, 1955), Coleoptera: Curculionidae)

Argentine stem weevil (ASW) originated in South America (Williams *et al.*, 1994) and was accidentally introduced to New Zealand and Australia where it has become a significant pasture pest. Due to the extreme damage caused by this insect it was the main target of early endophyte research in New Zealand (Prestidge *et al.*, 1982; Barker *et al.*, 1984a; Barker *et al.*, 1984b; Johnson *et al.*, 2013). Adults primarily feed on young seedlings and the adaxial surfaces of the leaves of agricultural grasses, creating distinctive 'window-like' rectangular feeding scars. Females oviposit under the outer sheath on the pseudostem and stem-boring larvae mine the centre of the tiller, moving between nearby tillers as they develop through four larval instars (Barker, 1988). Larvae are considered the most destructive life stage as they mine the youngest leaf sheath and often damage the apical meristem, resulting in tiller

death. Two to three generations are completed annually, with fewer generations developing in the cooler regions of the South Island, before this insect 'overwinters' (reproductive diapause) (Goldson, 1981) in the adult form. ASW are known to feed on maize, barley and wheat (Pottinger, 1961; Kain & Barker, 1966; Barker *et al.*, 1983).

Over the past two decades an integrated pest management strategy involving 'selected' fungal endophyte strains and a parasitic wasp (*Microctonus hyperodae* Loan, Hymenoptera: Braconidae) has been successfully implemented to help control this pasture pest (Goldson *et al.*, 1993; Goldson *et al.*, 2005; Popay & Hume, 2011; Johnson *et al.*, 2013; Ferguson *et al.*, 2018). Adult and larval feeding as well as oviposition are reduced by the naturalised CT endophyte and the 'selected' AR1 endophyte strains (Popay & Wyatt, 1995; Popay, 2000). Anti-feedant effects have primarily been attributed to the alkaloid peramine, which is produced by both strains and has been shown to reduce feeding of both adults and larvae when extracted and incorporated into a semi-synthetic diet (Popay *et al.*, 1990). This deterrent effect also reduces oviposition of adult ASW (Popay & Wyatt, 1995). In addition to peramine, the CT strain also expresses ergovaline and lolitrem B, of which ergovaline has been shown to reduce adult feeding and lolitrem B impacts on larval feeding only (Popay *et al.*, 1990). In comparison, the AR37 endophyte strain has no negative effect on adult feeding or oviposition but does significantly reduce damage caused by stem-boring larvae despite a lack of peramine expression (Popay & Wyatt, 1995).

In 1991, *M. hyperodae* was released in New Zealand to help control ASW (Goldson *et al.*, 1990; Goldson *et al.*, 1993). This South American wasp parasitizes adult weevils resulting in sterilization and eventual death (Loan & Lloyd, 1974). While this release was initially successful and ASW populations were significantly reduced, recent studies have reported a reduction in the rate of parasitism, suggesting that ASW adults may have evolved resistance to the parasitoid (Goldson *et al.*, 2014; Goldson & Tomasetto, 2016; Tomasetto *et al.*, 2018a; Tomasetto *et al.*, 2018b). Ferguson *et al.* (2018) estimated that the annual cost of ASW to agricultural production in New Zealand is between $160 and $200 million NZD, but this figure is likely to increase if ASW have developed resistance to *M. hyperodae*.

ASW larvae can move between tillers, but they are not thought to migrate far as they are susceptible to desiccation and predation. Therefore, the highly mobile adults are primarily involved in locating host plants, but the host-searching behaviour of this pest has not been explored in detail.

1.2.3 Perception of endophyte by host-searching insects

Studies investigating the interaction between agricultural grasses, endophyte and pest insects typically focus on outcomes such as the number of feeding scars or eggs laid on infected host plants (Barker *et al.*, 1984b; Jensen & Popay, 2004; Ball *et al.*, 2006; Jensen *et al.*, 2009; Popay & Thom, 2009; Thom *et*

al., 2013). These studies are important as they are used to evaluate bioactivity of naturalised and 'selected' endophyte strains. However, knowledge of these complex multitrophic interactions is incomplete as studies have not investigated how pasture pests perceive and subsequently avoid hosts infected with bioactive endophyte strains. In some interactions it is likely that perception is mediated by endophyte-derived alkaloids, but the underlying mechanisms involved in alkaloid perception are also not understood. Further insight could be gained by observing the effects of endophyte on the behaviour of host-searching insects and identifying when insects detect the presence of a bioactive endophyte strain in their host plant.

1.3 Host-plant searching, selection and acceptance

A phytophagous insect may need to search for a new host plant during migratory behaviour or when dispersing between primary and secondary host plants. Unfavourable conditions at their eclosion site, such as depletion of resources, overcrowding or increased predation can also force the insect to search for a new host plant. Locating a suitable host is vital as herbivorous insects are reliant on plants to complete their lifecycle and selection mistakes will lead to a loss of fitness in the adult and/or in its offspring.

Herbivorous insects gather information about host suitability before contacting the plant, through vision and olfaction and post-contact through contact chemoreception (taste) and mechanoreception (touch) (Thorsteinson, 1960; Bernays & Chapman, 1994; Bruce *et al.*, 2005; Schoonhoven *et al.*, 2005; Bruce & Pickett, 2011). Information gathered from these pre-ingestive mechanisms may result in the insect accepting the plant for sustained ingestion or in a rejection behavioural response.

1.3.1 Pre-contact cues

Visual stimuli

Visual stimuli may involve the colour of the plant or characteristics of its structure such as its size and shape. Aquatic milfoil weevils, for example, are able to differentiate between *Myriophyllum spicatum* (*Euhrychiopsis lecontei*, Coleoptera: Curculionidae), which has many leaflets and whorled compound leaves and *Ceratophyllum demersum*, which has whorled, single, needle-like leaves (Reeves & Lorch, 2009). The way in which insects respond to visual stimuli have been well studied in certain taxa such as the honeybee (Dyer *et al.*, 2008), bumblebee (Lunau, 1992; Spaethe *et al.*, 2001; Wilmsen *et al.*, 2017), aphids (Döring & Chittka, 2007) and flies (Green & Warnes, 1992; Drew *et al.*, 2006) but visual cues are often overlooked as important stimuli as 'all plants are green' (Schoonhoven *et al.*, 2005). Despite this, the majority of insect orders, including Homoptera and Coleoptera, have been shown to use visual stimuli for trap or plant detection (Reeves, 2011).

Evaluating insect response to visual stimuli

Responses to visual stimuli can be assessed in multi-arm and Y-type arenas as well as wind tunnels, locomotor compensators and sticky traps of different colours, shapes and sizes (Teulon *et al.*, 1999; Mayfield & Brownie, 2013; Arnold *et al.*, 2016; Yang *et al.*, 2017).

Study organisms

Visual responses of *A. lentisci* have not been assessed but clues as to their ability to perceive visual stimuli may be gained by examining the numbers of aphids caught on different traps in the field. O'Loughlin (1963) reported fewer *A. lentisci* in yellow water filled trays (n = 4) than on wind-vane traps (n = 32) in Victoria, Australia and a similar number of *A. lentisci* where found on green (n = 500) and yellow traps (n = 411) in a lettuce field in Murcia, Spain (Nebreda *et al.*, 2004).

Pottinger (1966) investigated visual discrimination of ASW adults in flight using different coloured traps (blue, black, red, green, white and yellow). More weevils were caught on yellow traps, closely followed by white and then green, demonstrating visual discrimination in this species. However, Pilkington (1987) later assessed visual orientation of ASW adults to coloured nutrient agar plugs (red, green, yellow, blue and white) and no preference was shown between any of the combinations. This suggests that colour discrimination is perhaps only important when ASW are in flight.

Olfactory stimuli

Plant volatiles

Plants are sedentary organisms and release volatile organic compounds from their leaves, stems, flowers, fruits and roots to interact with other organisms in their environment. Volatile blends can be extremely diverse and are comprised of terpenoids, fatty-acid derivatives, amino-acid derivatives and compounds with aromatic rings (Baldwin, 2010). Chemical signals can be highly valuable to the plant. Floral volatiles attract species-specific pollinators or seed dispersers and vegetative components release volatiles that can contribute toward defence (Pichersky & Gershenzon, 2002; Valenta *et al.*, 2017). Volatiles may be directly defensive or act indirectly by signalling the location of herbivorous insects to predators and parasitoids (Van Poecke & Dicke, 2004; Baldwin, 2010). Volatiles are also involved in plant-plant communication and may trigger neighbouring plants to prime their defences in preparation for insect attack (Baldwin *et al.*, 2002).

Phytophagous insects, however, have evolved highly sophisticated olfactory systems to take advantage of these diverse bouquets and are capable of detecting volatiles and herbivore induced plant volatile blends in the atmosphere and through the soil (Visser, 1986; Bruce *et al.*, 2005; Bruce & Pickett, 2011).

Insect olfaction

The primary olfactory organ of herbivorous insects are their antennae which are covered in small hair-like projections called chemosensilla which are involved in perception of volatile compounds (Figure 1.3). Sensillar can be in the shape of hairs, pegs, cones or plates and are characterised by the many pores present on the exoskeleton (Keil & Steinbrecht, 1984; Schoonhoven *et al.*, 2005). Although primarily housed on antennae, chemosensilla may be found on ovipositors and other head appendages including the maxillary palps (Wang *et al.*, 2012; Yadav & Borges, 2017).

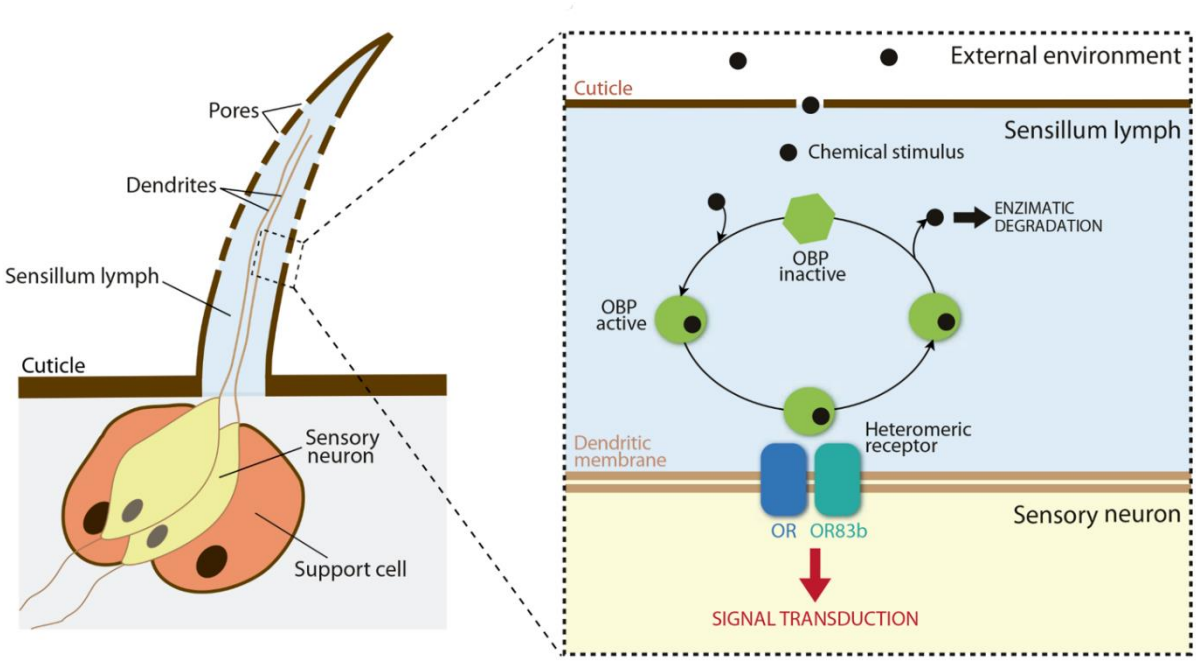


Figure 1.3: General structure of an olfactory sensilla and a simplified scheme depicting the first steps of the chemosensory signal transduction pathway. Reproduced with permission from Sánchez-Gracia *et al.* (2009).

Evaluating insect response to olfactory stimuli

Olfactory responses to plant odours are typically assessed in multi-arm olfactometers or Y-tubes which must be designed to suit the morphological and behavioural features of the test insect. For example, olfactometer requirements for subterrestrial larvae may include a substrate such as vermiculite, perlite or sterilised soil to simulate the conditions below ground. On the other hand a ladybird or aphid may require a linear wire to facilitate movement over the glass surface of a Y-tube (Hassall *et al.*, 2007; Rostás *et al.*, 2015). Olfactory responses may also be assessed in wind tunnels (Miller & Roelofs, 1978; Von Arx *et al.*, 2012), sophisticated locomotor compensators (Arnold *et al.*, 2016) or using electroantennography (EAG). EAG is a useful technique as it measures small voltage fluctuations across the antennae and can be used to determine whether an insect detects a specific compound or volatile

blend, but it does not provide information about the behavioural response elicited (Keesey *et al.*, 2012; Dekeirsschieter *et al.*, 2013; Balakrishnan *et al.*, 2017).

Investigating olfactory responses in a field situation is far more challenging given the small size of most insects. Field experiments typically involve mark, release and recapture studies or odour baited traps (Barros-Parada *et al.*, 2018; Venugopal & Subaharan, 2019). However, improvements in technologies such as high resolution laser-radar (lidar) or small, lightweight tracking devices will make it easier to track insects in the field in the future (Tahir & Brooker, 2011; Kirkeby *et al.*, 2016; Maggiora *et al.*, 2019). Behaviour can also be assessed in a 'semi-field' design by releasing an insect into an arena and observing its behaviour in relation to host and non-host plants (Pallini *et al.*, 1997; Dicke *et al.*, 2003).

Ryegrass volatiles

The odour blend released by the herbage of perennial ryegrass has been documented in previous studies but results were not consistent. Qawasmeh *et al.* (2015) reported 18 compounds in the volatile blend released by a perennial ryegrass cultivar in Australia and reported that the main components from endophyte-free plants were 2-ethylhexanol, dodecane, (Z)-2-hexen-1-ol and butyl hexanoate. In contrast, Pańka *et al.* (2013a) reported just eight volatiles emitted by three genotypes of endophyte-free and endophyte-infected perennial ryegrass collected from Poland and Austria. The main components in this blend were the two 'green-leaf' volatiles; (Z)-3-hexen-1-yl acetate and (Z)-3-hexenal as well as linalool. There is no doubt that further investigation of the volatile profile emitted by perennial ryegrass is required.

Study organisms

Olfactory responses of *A. lentisci* to perennial ryegrass have not been assessed but Wool *et al.* (1994) noted that alatae sexuparae of this species made a number of landing errors on the wrong species of *Pistacia*, suggesting perhaps that this aphid's use of pre-contact cues is not as efficient as it is in other related aphid species. Olfactory responses of ASW have been investigated. Pilkington (1987) identified no chemotactic behaviour in a four-arm-olfactometer with dynamic airflow (Vet *et al.*, 1983), but in an unpublished study ASW were shown to respond positively towards damaged Italian ryegrass in a still-air olfactometer (J. Vereijssen, personal communication).

Specificity in pre-contact cues

Olfactory cues are generally considered to be more decisive factors in host plant selection and acceptance behaviours than visual cues (Schoonhoven *et al.*, 2005). The diversity of visual cues is restricted by both the light spectrum and the limited diversity of photoreceptor types and visual pigments in insects. This is in direct contrast to the endless diversity of olfactory blends that can derive from the plethora of plant volatile organic compounds. As a result, olfactory cues are considered to be more specific than visual cues.

1.3.2 Post-contact cues

The surface is the first plant feature that an insect encounters after landing. The physical features of the surface such as trichomes, wax crystal structures, toughness and thickness can impact host-selection and this information is likely coded by mechanosensory sensilla found on various appendages (Ramaswamy, 1988; Schoonhoven *et al.*, 2005). However, behavioural responses to physical features cannot account for the high degree of specialization shown by herbivorous insects (Schoonhoven *et al.*, 2005). Taxonomic patterns observed in plant chemistry far exceed any patterns in physical features and possibly provide the basis for host-plant specificity by phytophagous insects (Schoonhoven *et al.*, 2005).

Contact chemoreception

Contact chemoreception is often described as an insect's 'sense of taste'. However, the terms 'contact chemoreception' or 'gustation' are more appropriate when discussing this mechanism as receptors are not only found on the mouthparts but also the tarsi, antennae and even the ovipositor (Figure 1.4) (Chapman, 2003). The primary function of gustatory receptors are to enable food recognition. Thus, receptors can detect primary metabolites such as carbohydrates and amino acids as well as secondary metabolites such as plant alkaloids (White *et al.*, 1990; Chyb *et al.*, 1995; Schoonhoven & Van Loon, 2002; Chapman, 2003).

Contact chemoreceptors are typically in the shape of hairs or cones and, unlike olfactory receptors, have a single pore at their terminus. Some insect taxa can have a large number of contact chemoreceptors. For example, adult *Locusta migratoria*, a migratory locust, are thought to have 3,000 gustatory receptors on their mouthparts alone (Chapman, 2008). Gustatory receptors, like olfactory receptors, generate action potentials after suitable compounds enter the sensillum and spikes travel to the local segmental ganglion or the suboesophageal ganglion which is where the motor neurons of the mandibular muscles are housed. Information from other gustatory receptors as well as mechanoreceptors and internal receptors are integrated and, depending on the information received, feeding may or may not occur (Schoonhoven *et al.*, 2005). When compared to the olfactory system, less is known about the central integration of the information received by these receptors and unlike olfactory signals, which converge in the glomeruli, information does not appear to converge in a specific area in the central nervous system (Schoonhoven *et al.*, 2005).

After an insect makes contact with a plant it may begin to explore the surface by performing behaviours such as antennation, palpation, tarsal drumming and scratching (Chapman & Sword, 1993; Headrick *et al.*, 1996). These behaviours often involve repetitive, rapid movements the purpose of which is to bring chemoreceptors on appendages into contact with plant material. This contact provides the insect with information about the composition of the dry surface of the leaf. Insects may

then ‘bite’ or ‘macerate’ plant material, releasing plant fluids that immerse chemoreceptors around the mouthparts and allow the insect to detect compounds that occur in solution. An insect may utilise the information it receives from sensory stimuli alone to decide whether to accept or reject the plant as a suitable host for feeding or oviposition (Chapman & Bernays, 1989; Schoonhoven *et al.*, 2005).

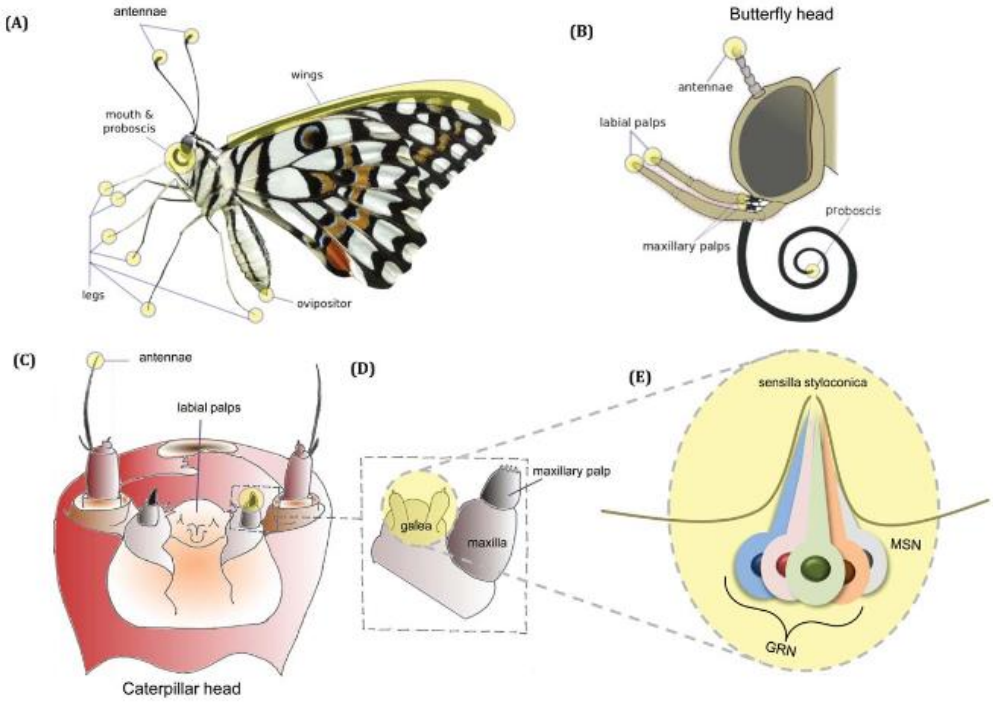


Figure 1.4: Locations of contact (gustatory) chemoreceptors on a Lepidopteran adult (A - B) and caterpillar head (C - E). GRN = gustatory receptor neuron. MSN = mechanosensory neurone. Reproduced with permission from Agnihotri *et al.* (2016).

1.3.3 Post-ingestive mechanisms of detection

There are cases where behavioural rejection appears to occur after a period of feeding, indicating a post-ingestive mechanism rather than sensory deterrence (Glendinning, 1996; Renwick *et al.*, 2001; Glendinning, 2002). An example of this is the rapid post-ingestional rejection demonstrated in naïve larvae of *Manduca sexta* (Lepidoptera: Sphingidae) (Glendinning, 1996). Larvae initially feed on a nicotine-containing diet but stop abruptly (within 30 seconds), after which time some larvae were observed twitching and writhing. The authors postulated that a post-ingestive response mechanism was involved as ablation of mouthpart chemoreceptors did not alter the rejection response and sensory recordings did not indicate a plausible role of gustatory sensilla (Glendinning, 1996). Furthermore, gustatory responses in experienced *M. sexta* are typically quicker (< 6 seconds) and twitching is a sign of nicotine entering the central nervous system (Glendinning, 1996, 2002). A similar response was demonstrated in fall armyworm caterpillars (*Spodoptera frugiperda*, Lepidoptera:

Noctuidae) in response to the compound indole 3-carbinol. Larvae were observed to feed for two to three minutes before they stopped and became motionless (Glendinning & Slansky, 1995). Results were indicative of a post-ingestive mechanism as larvae were not deterred from initiating feeding. Renwick *et al.* (2001) also suggested a post-ingestive mechanism for response to a compound in the plant *Alliaria petiolata*. Neonate larvae of the American butterfly (*Pieris napi oleracea*, Lepidoptera: Pieridae) appeared to feed within a four-hour period, as green material could be seen in the gut of translucent larvae, before the insect stopped feeding and became motionless. It is important to consider whether an insect is deterred pre or post-ingestionally as an insect may cause a significant amount of damage before it is deterred from feeding.

1.3.4 Influence of endophytes on selection behaviour

When investigating multitrophic interactions it is important to have an understanding of the external abiotic and biotic factors which may influence the interaction as these factors may override the behavioural responses which are observed under standard conditions (Bernays & Chapman, 1994). Plant-associated microorganisms are becoming increasingly recognised as an important factor to consider when investigating plant-insect interactions as common plant viruses, diseases and fungi are known to alter plant chemistry and consequently the attractiveness of the plant. An example comes from experiments with *Rhopalosiphum padi* (Hemiptera: Aphididae) which were found to preferentially congregate on screens located above wheat plants infected with the barley yellow dwarf virus rather than wheat plants not infected with the virus (Jiménez-Martínez *et al.*, 2004). Experiments were carried out in the dark and suggested that *R. padi* responded to changes in the plant volatile blend.

Fungal endophytes of grasses have been studied extensively and are well known to alter the chemistry of the host plant (Johnson *et al.*, 2013). Endophyte derived secondary metabolites are frequently cited in the literature, but in addition to these compounds infection has been shown to affect the available nutrients and sugars and has recently been shown to alter the plant volatile blend (Gallagher *et al.*, 1981; Ball *et al.*, 1997a; Leuchtmann *et al.*, 2000; Rasmussen *et al.*, 2012; Saikkonen *et al.*, 2013; Vázquez-de-Aldana *et al.*, 2013; Rostás *et al.*, 2015). Numerous studies have demonstrated that endophyte-infection can alter whether a host plant is accepted for sustained feeding and egg laying by caterpillars, beetles (including weevils) and aphids (Popay & Wyatt, 1995; Popay & Baltus, 2001; Pennell *et al.*, 2005; Jensen *et al.*, 2009; Johnson *et al.*, 2013; Young *et al.*, 2013; Ruppert *et al.*, 2017). However, these studies report outcomes (e.g. number of feeding scars or eggs) and do not investigate the effect of endophyte-derived compounds on insect behaviour and in particular the behaviour leading up to host plant acceptance.

Two recent studies have reported that African black beetle adults and grass grub larvae (*Costelytra giveni*, Coleoptera: Scarabaeidae) orient towards the volatile blend released by endophyte-free host plants when presented with a choice between these plants and plants infected with endophyte (Qawasmeh *et al.*, 2015; Rostás *et al.*, 2015). This is an interesting finding as it indicates that certain insects may be able to detect the presence of endophyte within their host plant before they have contacted plant material. This research is, however, in the early stages and further studies are required to assess whether all pasture pests are capable of perceiving endophyte using olfaction and whether these responses are seen in the field where insects are exposed to a suite of other stimuli.

1.4 Outline and aims

Despite 40 years of research into *Epichloë* fungal endophytes of grasses there is still much we do not know. A major gap in our knowledge is understanding how insects perceive and subsequently avoid bioactive endophyte strains in perennial ryegrass. In this thesis studies were designed to investigate olfactory and behavioural responses of two major pasture pests to endophyte-infected (*Epichloë festucae* var. *lolii*, various strains) and endophyte-free perennial ryegrass host plants. Since rejection was thought to involve a likely chemical basis, experiments explored (1) olfaction, (2) contact chemoreception and (3) post-ingestive effects. Where appropriate, experiments were designed to investigate combined effects of endophyte and herbivory as both factors are known to have a significant effect on plant chemistry. Volatile compounds were collected, identified and quantified using gas-chromatography mass-spectrometry to investigate the volatile blend and to identify differences between endophyte-infected, endophyte-free and herbivore damaged plants.

This research will increase our knowledge of the chemical basis of endophyte-mediated defence and provide an understanding of the behavioural responses of two major pests to bioactive endophyte strains. Determining how endophyte (endophyte-derived compounds) are detected by host-searching insects is challenging due to the complexity involved in these multitrophic interactions. However, a greater understanding will ensure that these mechanisms are conserved when selecting new endophyte strains for commercialisation. Results from this study could have broader applications, as naturally occurring behavioural responses are expected to be identified and these could be exploited to develop novel crop management strategies in the future. Such strategies may include trap cropping, host-masking, lures and deterrent odour blends.

Chapter 2

Olfactory and Behavioural Responses of *Aploneura lentisci* to Perennial Ryegrass (*Lolium perenne*)

2.1 Abstract

Aploneura lentisci (root aphid) is a host-alternating aphid in its native range but in New Zealand, where the primary host is not found, populations of apterous parthenogenetic aphids occur year-round on the roots of agricultural grasses. The predominant grass species in New Zealand, perennial ryegrass (*Lolium perenne*), forms a defensive mutualistic association with the fungal endophyte, *Epichloë festucae* var. *lolii*. Endophyte-free perennial ryegrass or ryegrass infected with certain strains (genotypes) of this endophyte are suitable host plants for this pest. However, the AR37 endophyte strain has a potent effect on this aphid. Olfactometer bioassays and a host preference assay were carried out in this study to investigate host-searching, selection and acceptance behaviour of apterous *A. lentisci*. When presented with a choice between endophyte-free and AR37-infected perennial ryegrass *A. lentisci* colonised plants equally and thus were unable to initially (24 h) perceive endophyte, demonstrating that negative effects of endophyte are not always associated with initial perception and avoidance responses. The significance of this result is discussed. A still-air olfactometer design was chosen and methodology developed to evaluate olfactory responses of *A. lentisci* nymphs, as this highly mobile life stage is thought to be primarily responsible for dispersal of clones between secondary host plants in New Zealand. Apterous nymphs did not move towards the roots or herbage of their host plant in olfactometer experiments, indicating that under the experimental conditions I used apterous *A. lentisci* do not utilise olfaction during host-searching. Olfactory responses of above ground aphids have been well studied but this appears to be the first study to investigate olfactory responses of a below ground aphid.

2.2 Introduction

The asexual fungal endophyte *Epichloë festucae* variant *lolii* (Latch, M. J. Chr. & Samuels, Hypocreales: Clavicipitaceae) forms a defensive mutualistic association with perennial ryegrass (*Lolium perenne* Linnaeus, Poales: Poaceae), the predominant agricultural grass species in New Zealand (Clay, 1988; Saikkonen *et al.*, 2010). Endophyte hyphae colonise above ground tissues of host plants and are transmitted to the next generation within the seed of the host plant (Johnson *et al.*, 2013). This interaction provides host plants with protection against phytophagous pest insects and is essential for pastoral farming in New Zealand, where a scarcity of natural predators has allowed introduced and native phytophagous pests to flourish (Prestidge *et al.*, 1982; Johnson *et al.*, 2013; Ferguson *et al.*,

2018). Pest protection has largely been attributed to the presence of bioactive alkaloids and although hyphae are only present in aerial sections of the plant some of these compounds are translocated into the roots where they can alter below ground insect communities (Patchett *et al.*, 2008; Bryant *et al.*, 2010; Patchett *et al.*, 2011).

The root aphid, *Aploneura lentisci* (Passerini, 1856; Hemiptera: Aphididae), infests the roots of perennial ryegrass and reduces plant vigour by sucking sap from the phloem. Until recently, *A. lentisci* had not been considered a significant pest as it was poorly understood and the small size, fragility and sensitivity of *A. lentisci* make it a difficult insect to study (Popay & Cox, 2016; Müller, 2019). As a result, its impacts are often incorrectly attributed to other pasture pests or abiotic conditions such as drought and in a recent review of New Zealand's pasture pests Ferguson *et al.* (2018) could not assess the pest status and economic importance of this aphid. Although the effects are transitory, the naturalised common-toxic endophyte strain (genotype), commonly found in older pastures in New Zealand, can have some effect on *A. lentisci*, but the 'selected' AR1 strain has no negative effect (Hume *et al.*, 2007; Popay & Gerard, 2007; Popay & Thom, 2009). In contrast, the AR37 endophyte strain has a potent effect on *A. lentisci* which results in low aphid populations and increased growth of plants containing this endophyte (Pennell *et al.*, 2005; Popay & Gerard, 2007; Popay & Cox, 2016). Assessments of mortality and aphid population sizes have been conducted to ascertain which endophyte strains are effective against this species, but it is not known whether *A. lentisci* can perceive the bioactive endophyte strain (AR37) before succumbing to a potent neurotoxin (Popay & Cox, 2016). If this endophyte strain is shown to be perceived by host-searching aphids it would then be of interest to ascertain the mechanisms involved.

A. lentisci is endemic to the Mediterranean where it has a complex two-year holocyclic life cycle alternating between galls on the leaves of its primary host, *Pistacia lentiscus* and the roots of Poaceae (Wool & Manheim, 1986, 1988; Wool, 1995). The primary host is not found in New Zealand and parthenogenetic populations of apterous root aphid occur year round on the roots of agricultural grasses (Popay & Cox, 2016). Mature aphids are largely sedentary and remain attached to a root system, producing a layer of white flocculent wax for protection. In contrast, nymphs are highly mobile and have been recorded above ground on the herbage of host plants (Rasmussen *et al.*, 2008). Although alatae aphids were found by Lowe (1968) in grease traps established in Canterbury, New Zealand, they have not been observed when sampling below ground populations in the field. Therefore it is not known whether alatae morphs are required for dispersal of clones among secondary host plants in New Zealand's pastoral ecosystems (Popay & Cox, 2016). It has been hypothesised that the highly mobile apterous nymphs are primarily responsible for dispersal in New Zealand and are therefore responsible for host-selection and acceptance (Popay & Cox, 2016).

Aphids are a significant pest of agricultural and horticultural crops around the world. Infestations can result in large economic losses due to feeding damage and transmission of viruses (Ng & Perry, 2004). There is considerable interest in determining how different aphid species locate their host, as insights can provide opportunities for control (Döring, 2014). Host plant selection can be divided into 'host-finding' and 'host acceptance' (Thorsteinson, 1960; Finch & Collier, 2000). In general, the initial stage of aphid host-finding (host-searching) often involves detection of visual (e.g. colour) and/or olfactory (e.g. volatile organic compounds) signals released by the plant (Döring & Chittka, 2007; Webster, 2012). Aphids will then make contact with the plant material and gather olfactory and gustatory cues as well as visual and textural cues from the surface (Neal *et al.*, 1990; Rodriguez *et al.*, 1993; Storer *et al.*, 1996; Powell *et al.*, 1999). An aphid may then probe the plant material several times. If positive signals are obtained the aphid will insert its stylet deeper and eventually into the phloem sieve element (Tjallingii, 1995; Powell & Hardie, 2000, 2001). Acceptance occurs after the aphid has made contact with, and fed from, the phloem for an extended period (Powell *et al.*, 2006). Host-searching behaviours can differ between species and between apterous and alatae morphs of the same species (Bernasconi *et al.*, 1998; Quiroz & Niemeyer, 1998). Knowledge of host-searching behaviour in aphids is largely based on above ground species. Studies on root aphids are uncommon and little is known.

In this chapter I explored whether mature apterous aphids could perceive endophyte. I hypothesised that, when searching for, and selecting a host in their immediate environment, probing aphids would perceive the AR37 endophyte strain and select the more favourable endophyte-free plant. I then explored the role of olfaction in host-searching behaviour of highly active apterous *A. lentisci* nymphs, which may be involved in dispersal of the clone. I investigated olfactory responses to both the roots and herbage of the host plant as it is not known whether this species disperses above or below ground. Gaining a basic understanding of how *A. lentisci* locate and then colonise new host plants in New Zealand will add to our very limited knowledge and understanding of this pasture pest and may lead to the identification of novel management strategies in the future.

2.3 Methods

2.3.1 Establishment of ryegrass plants and endophyte testing

Endophyte-infected (AR37) and endophyte-free perennial ryegrass plants (*Lolium perenne* cultivar 'Grasslands Samson') were established from seed obtained from the Margot Forde Germplasm Centre, AgResearch (Palmerston North, New Zealand). Seeds were germinated at 20°C in Petri dishes (90 mm) lined with damp filter paper. Germinated seedlings were planted into forestry trays containing a commercial potting mix (Daltons™ - New Zealand pine bark fines and fibre, pumice, coco fibre, controlled release fertilizer and a wetting agent) and left to establish. Plants were watered and trimmed as required. All plants were tested for endophyte infection using a tissue print immunoassay.

One tiller per plant was cut within 5 mm of the base of the plant where endophyte mycelia are concentrated. Dead sheath material was removed and the cut surface was pressed firmly onto a sheet of nitrocellulose paper (Amersham™ Protran™ 0.45 µm NC). Endophyte blot sheets were developed following an immunoassay protocol described by Simpson *et al.* (2012). Incubation and washing methods were altered slightly to improve endophyte detection. The primary antibody was shaken overnight while incubated at 4°C and the secondary antibody was shaken for 2 hours at room temperature. The washing method was altered by adding a two-minute shaking step and the number of rinses were increased from two to five. In addition, a new primary antibody (Rabbit anti – AR93 endophyte) was produced from a bulk bleed (Lyn Briggs and Jan Sprosen, AgResearch Ruakura). Immunoassays were carried out by Jan Sprosen (AgResearch).

2.3.2 *Aploneura lentisci*

Mature *A. lentisci* (all apterae) were collected from the roots of potted ryegrass plants (Figure 2.1) that were maintained in a shadehouse. A fine tipped paint brush was used to agitate the aphid slightly and to ensure removal of the stylet from the plant root before the aphid was moved. Mature *A. lentisci* (3 – 4 individuals per vial) were placed into Eppendorf tubes (2 mL) with a small amount (3 – 4 pieces of root approximately 2 mm in length) of grass root (AR1-infected ryegrass was used with the exception of experiment 1 rep 9 where half of the colony was exposed to root material from endophyte-free meadow fescue plants due to a shortage of fresh root material). AR1-infected ryegrass was chosen over endophyte-free ryegrass as AR1 does not negatively impact root aphid and population sizes found on AR1-infected plants often exceeds those found on endophyte-free ryegrass (Pennell *et al.*, 2005; Popay & Gerard, 2007). To provide a sufficient number of aphids for the olfactory experiments a colony consisting of 30 - 60 mature aphids was established weekly which provided 30 - 60 nymphs each week. Nymphs were no more than 7 days old (except in replicates 11 and 12 of the herbage experiment where aphids may have been up to 12 days old) and were not considered to be naïve as they had prior exposure to root material in Eppendorf tubes. Mature aphids used in the gustatory choice test were collected fresh from potted plants (AR1-infected ryegrass) the day before each experiment and held in Eppendorf tubes with grass roots overnight (AR1-infected ryegrass, 2 x 2 mm strips). Eppendorf tubes were stored in a rack which was covered in tin foil and kept in a 20°C controlled environment chamber. This ensured that only healthy aphids were selected for the experiment.

Figure 2.1: Mature *Aploneura lentisci* (yellow) surrounded by protective wax (white) attached to the root system of a potted ryegrass plant (*Lolium* sp.).

2.3.3 Host preference assay

A host preference assay was run to investigate whether mature *A. lentisci* select their preferred endophyte-free perennial ryegrass hosts over ryegrass infected with the bioactive AR37 endophyte strain (Figure 2.2). Two or three tillers (with root material attached) were split from each parent plant and re-potted into small containers (65 mL) filled with topsoil and left to establish in a glasshouse. Both the roots and herbage of each plant were trimmed to 9 cm and plants grown for 5 - 7 days to allow the plant to recover and to establish new roots that are thought to be highly attractive to root aphid (Popay & Cox, 2016). A draught stopper was placed around the outer edge of a glass sheet (260 mm x 160 mm). Fresh damp soil was sieved and lightly compacted onto the bottom half of the glass (120 mm). There was sufficient space between the soil surface and the top glass sheet to allow aphids to move around without restriction. For each of the eight replicates an endophyte-free and an AR37-infected plant were removed from their containers and the roots lightly compacted on top of the soil side by side on the glass sheet. Plant herbage was laid over the top half of the glass sheet. Fifteen mature *A. lentisci* were placed on the soil surface, in between the plants, near to the bottom of the glass sheet. A second glass sheet was placed over the top and the apparatus tilted against a hard surface so that the bottom of the glass sheet was 130 mm from the wall. Dark cloth was placed over the experiment to exclude light and the experiment was run at approximately 20°C. After 24 hours the experiment was deconstructed, and the position of each aphid recorded (endophyte-free plant roots, AR37-infected plant roots and test arena (i.e no-choice)). Glass sheets were cleaned between replicates with hot water. Plant position (left or right) was orientated randomly.

Figure 2.2: *Aploneura lentisci* presented with a choice between an endophyte-free and an AR37-infected perennial ryegrass (*Lolium perenne*) plant. Arrow indicates the position that root aphid were released into the arena.

2.3.4 Olfactometer

A number of olfactometers were constructed and trialled by observing and recording *A. lentisci* behaviour and movement. Based on these observations a linear design was chosen. The 'Root Aphid Olfactometer' (Figure 2.3) was produced out of glass by Greg Purdy, Glasslab, Hamilton, New Zealand. The olfactometer consisted of two glass cups (70 mm height x 50 mm diameter) connected by a cylindrical central chamber (180 mm length x 15 - 19 mm diameter). The central chamber consisted of three pieces of equal length (60 mm) - two arms and a middle chamber. Each arm was fitted with a glass sinter (Duran® sintered glass filter, nominal maximum pore size 160 - 250 µm, I.d. ISO 4793 P250) which allowed for the diffusion of volatile organic compounds from odour source cups into the central chamber, while preventing *A. lentisci* from accessing plant material. The middle chamber contained an opening, covered by a glass stopper, which allowed test insects to be added to the central chamber. Neonate aphids were found to be very sensitive to light, humidity, moisture and temperature. Experiments were performed to determine the best protocol for this combination of factors.

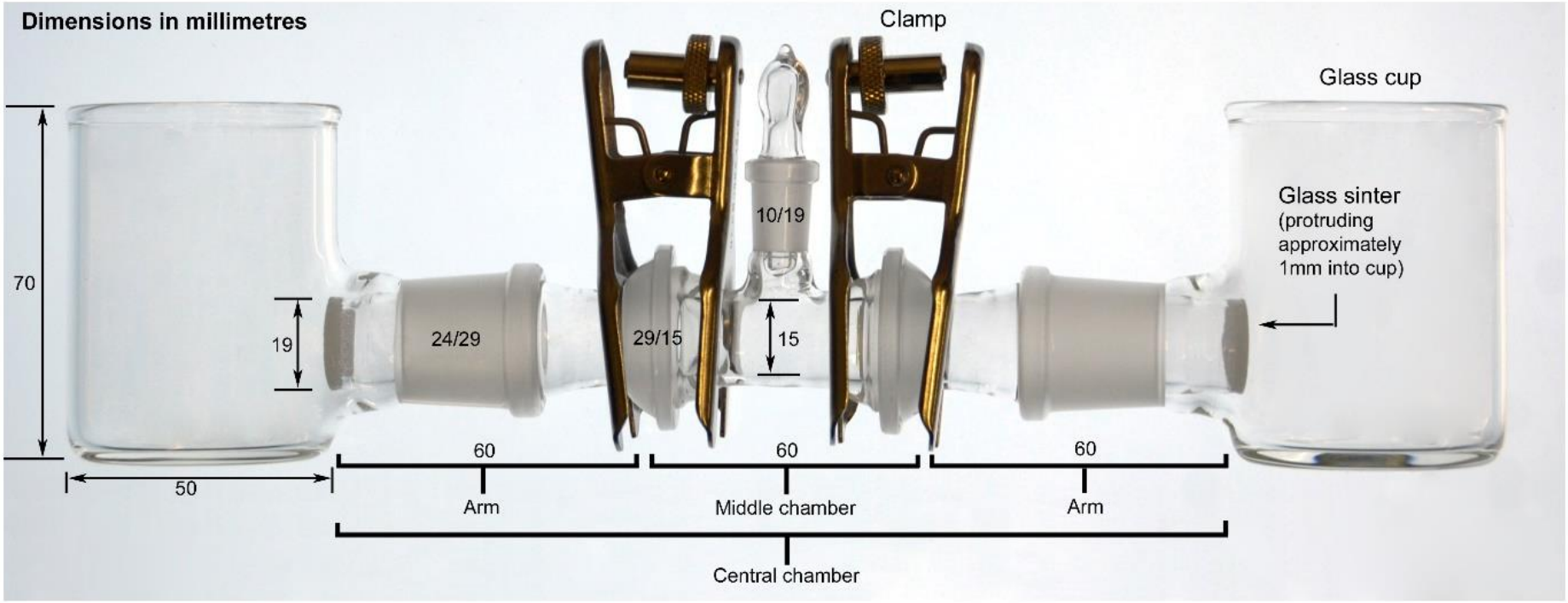


Figure 2.3: 'Root Aphid Olfactometer' with measurements (mm). Photographed by Greg Purdy, Glasslab, Hamilton, New Zealand.

2.3.5 Olfactory responses to endophyte-free host root volatiles

Response to root volatiles - Experiment 1

An olfactory experiment was carried out to investigate whether apterous *A. lentisci* nymphs were attracted to the volatile blend released by the roots of their host plant. Endophyte-free ryegrass plants were removed from forestry trays and potting mix rinsed from the roots. Both the herbage and roots of each plant were trimmed to 6 cm (27 – 40 days before the experiment). Plants were re-potted into individual mesh containers (90 mm height x 50 mm diameter) with sieved perlite (2 mm). Mesh containers were placed into tin foil cups (80 mm height x 60 mm diameter) to protect roots from the light and to prevent the perlite from drying out. Plants were maintained in a glasshouse, watered with 15 mL of tap water as required and fertilized with 10 mL of nutrient solution (General hydroponic Flora Nova Grow, 3 mL to 997 mL cold tap water) once a week.

To set up an olfactometer, a ryegrass plant was re-potted into one of the glass cups with sieved damp perlite (5 g perlite + 7 mL of cold tap water). The second glass cup was lightly packed with damp perlite (5 g + 7 mL) to act as a control and the central chamber was filled with dry perlite (Figure 2.4). The olfactometer was wrapped in tin foil leaving the plant herbage exposed to the light and was placed into a controlled environment chamber (19°C, 80% humidity) for two hours to allow volatile chemicals to diffuse. Ten neonate *A. lentisci* were starved (1 - 1.5 h) before being placed into the middle of the central chamber. After two hours the olfactometer was deconstructed and the number of aphids in each section counted and recorded. Ten replicates were run although replicates 3 and 4 contained 9, rather than 10 aphids. Between replicates the olfactometer was washed with hot soapy water, rinsed with MQ water, wiped out with drum ethanol and left to bench dry for at least 30 minutes. Plant position was orientated randomly.

Response to root volatiles - Experiment 2

In an attempt to improve aphid response, the method described above was modified. Plants were placed in the controlled environment room for a greater time period (5 h) and aphids were starved for longer (3 h). In addition, the perlite was damper (10 g sieved perlite + 20 mL cold tap water) and the glass cups were covered with tin foil to reduce moisture loss. Plants were fertilized 10 – 13 days (previous experiment was between 3 and 8 days) before inclusion in an experiment as it had been suggested that fertilisation may negatively affect this root aphid. All plants were re-potted into mesh containers exactly 7 days before the experiment to ensure fresh root growth.

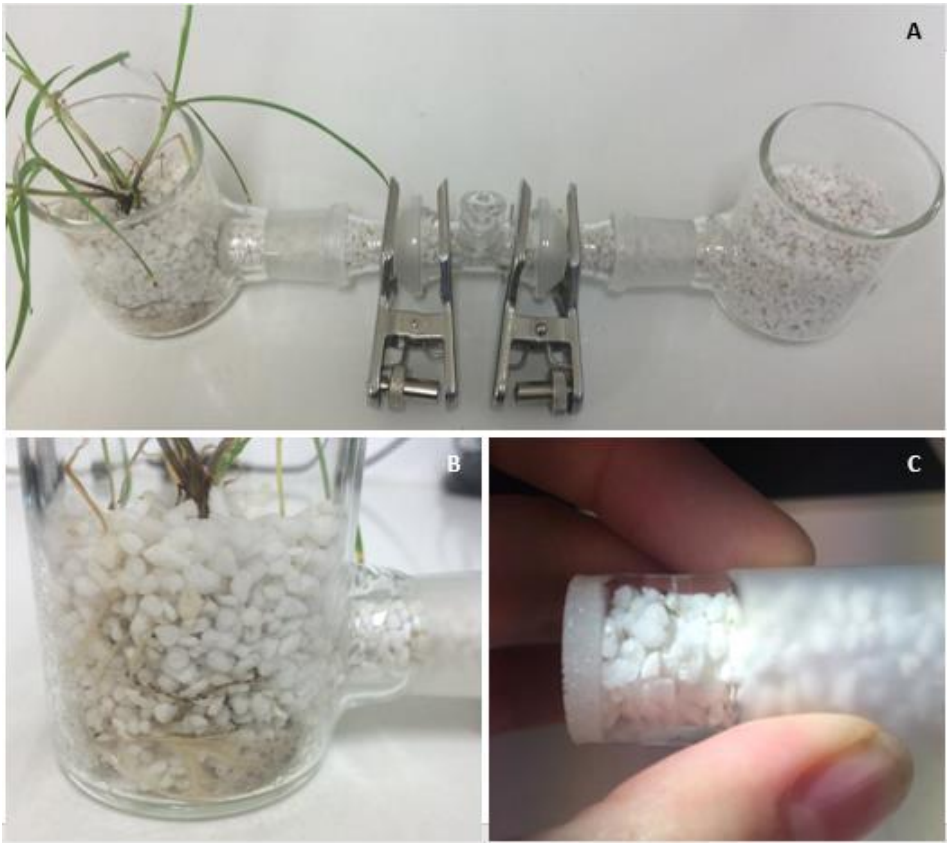


Figure 2.4: 'Root Aphid Olfactometer' filled with perlite. **A)** whole olfactometer with a perennial ryegrass (*Lolium perenne*) plant in one cup and damp perlite in the other. **B)** roots of a perennial ryegrass plant in the glass cup of the olfactometer. **C)** arm of the olfactometer showing the glass sinter.

2.3.6 Olfactory responses to endophyte-free host foliar volatiles

The aim of the third olfactometer experiment was to investigate whether aphids were attracted to the volatile blend released by the herbage of their host plant (Figure 2.5). Parafilm® (flexible film) was used to seal the top of both cups. An endophyte-free perennial ryegrass plant was removed from its container and the roots placed into a zip lock plastic bag which was sealed around the crown of the plant. A small hole was cut in the Parafilm® through which the plant herbage was pushed into the cup. The zip lock bag containing the plant roots sat on top of the Parafilm® to limit contamination from root volatiles. Absorbent cotton wool (630 mg) was placed in the second glass cup with 1 mL of cold tap water to keep the moisture constant between the two treatments. Humidity in each cup was measured and recorded at the beginning of each experiment (McGregors weather station/thermometer). The central chamber was not filled with a substrate. The olfactometer was left for 40 minutes to allow volatile compounds to diffuse through the central chamber. Fifteen apterous nymphs were placed in the middle of the central chamber and left for 1 h 15 min to 'make a choice'. The olfactory experiment

was run in the dark (to exclude visual cues) on a lab bench where the temperature was approximately 20°C. Two replicates were run per day with 15 aphids in each replicate olfactometer and a total of 12 replicates were completed. Plant position (left or right) was randomised between replicates.

Figure 2.5: 'Root aphid olfactometer' to investigate attraction of *Aploneura lentisci* to the volatile blend released by perennial ryegrass herbage (*Lolium perenne*). Aphids where placed in the central chamber and presented with a choice between perennial ryegrass and a control of damp cotton wool.

2.3.7 Statistical analyses

Aphid location data was analysed using an Analysis of Variance, blocked by replicate (GenStat 18th edition). Variables were not transformed. Differences were compared using Fisher's Unprotected least significant difference *post hoc* tests, conducted at the 5% significance level.

2.4 Results

2.4.1 Influence of endophyte on *Aploneura lentisci* host preference

Aploneura lentisci were given a choice between endophyte-free and AR37-infected perennial ryegrass which allowed aphids to gather sensory information as well as probe and feed on plant material. No significant difference ($P = 0.947$, $F_{2, 14} = 0.05$) in aphid host-selection was found (Figure 2.6) as equal numbers of aphids were found on AR37-infected (average 5.1 aphids) and endophyte-free (average 4.8) ryegrass plants. Observations were not made on whether aphids were feeding on the roots. Overall mortality in this experiment was low (1.7%) and the average response rate (number of aphids that selected a host plant) was 68%.

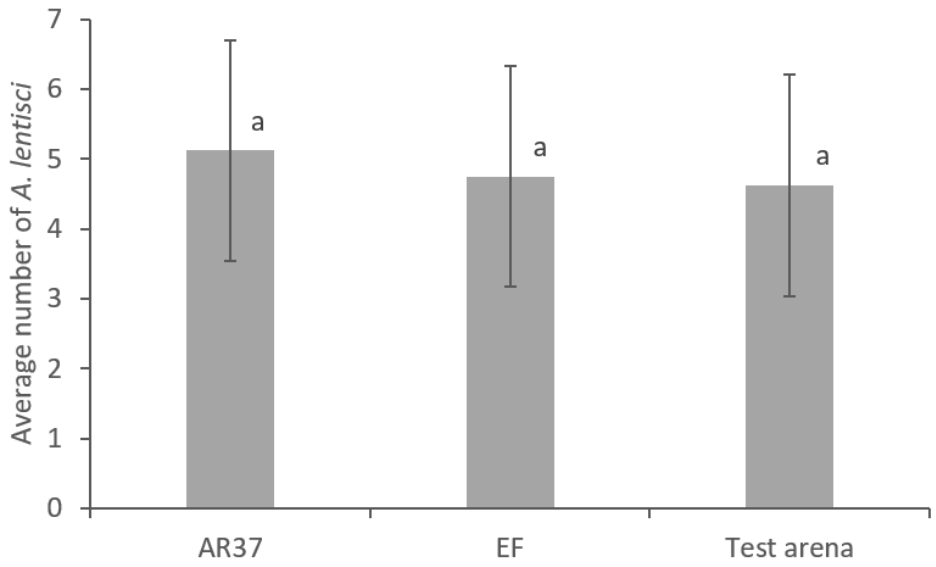


Figure 2.6: Average number of *Aploneura lentisci* found on the roots of AR37-infected perennial ryegrass (*Lolium perenne*), endophyte-free (EF) perennial ryegrass and aphids that did not make a choice (test arena) in the host preference assay (n = 8). +/- s.e.d [standard error of the difference]. Letters denote significant differences between treatments; ANOVA, $P < 0.05$.

2.4.2 Olfactory responses to endophyte-free host root and foliar volatiles

In root olfactometer experiments *A. lentisci* nymphs were presented with the choice of orientating themselves towards perennial ryegrass roots or a control of damp perlite (Figure 2.7A and B). In contrast to my hypothesis that the volatiles released by ryegrass roots would attract aphids, more *A. lentisci* selected the control over the host plant, a difference which was statistically significant in Experiment 1 ($P = 0.017$, $F_{2,18} = 5.19$). The average response rate was 65% in Experiment 1 (Figure 2.7A), but only 39% in Experiment 2 (Figure 2.7B). Overall mortality in both experiments was low (6.3% and 3.4%).

When aphids were given the choice of orientating themselves towards the volatile blend released by ryegrass herbage or a control of damp cotton wool no significant ($P = 0.137$, $F_{2,22} = 2.18$) difference was observed (Figure 2.7C). The response rate in this olfactometer experiment was higher (74%) than that of the root experiments and overall mortality was equally low (4.0%).

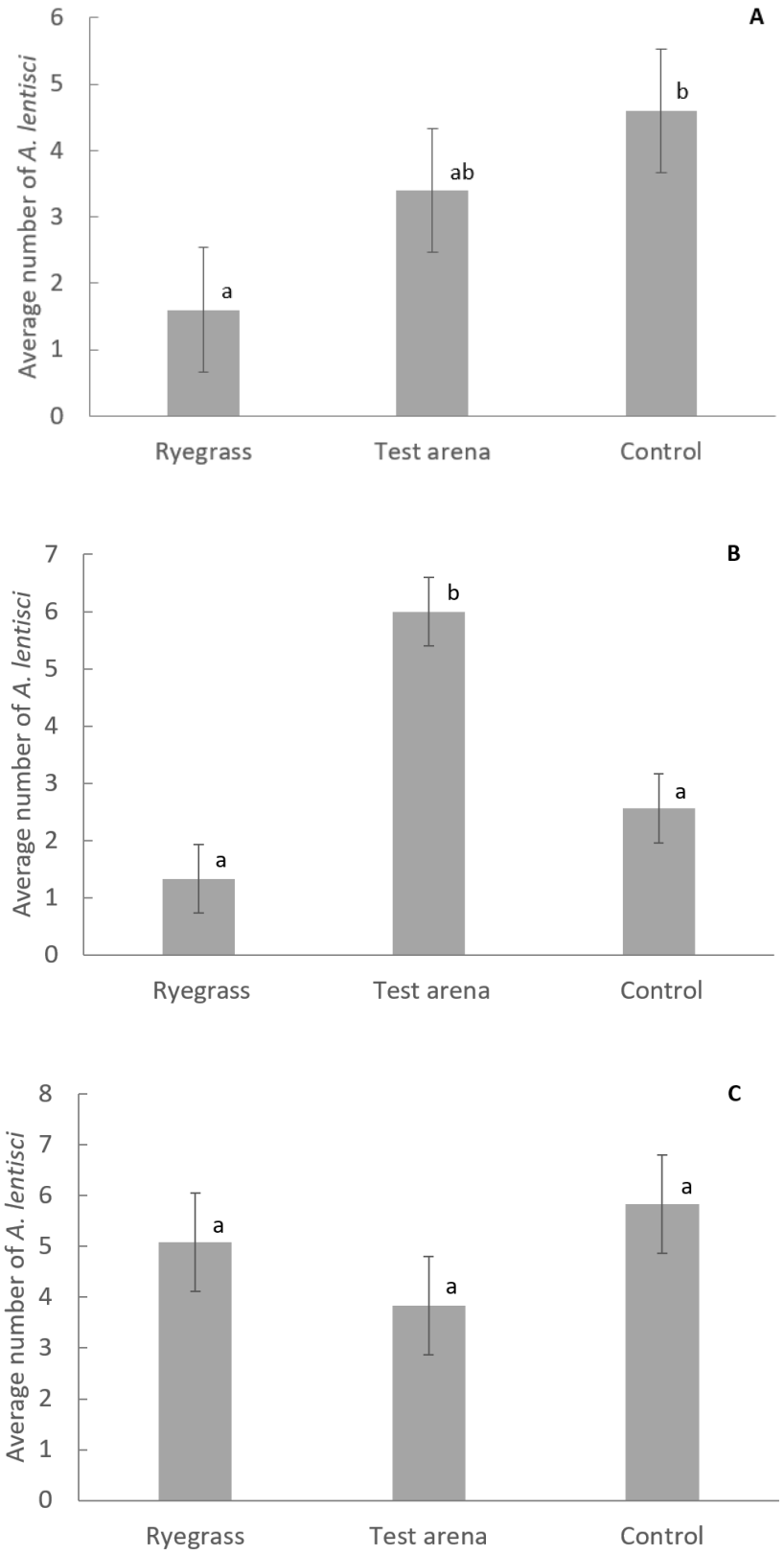


Figure 2.7: Average number of *Aploneura lentisci* that moved towards the endophyte-free perennial ryegrass (*Lolium perenne*) plant, perlite control or remained in the test arena (central position where aphids where added) in olfactometer bioassays; **A)** root olfactometer Experiment 1 (n = 10), **B)** root olfactometer Experiment 2 (n = 9), **C)** herbage olfactometer experiment (n = 12). +/- s.e.d. Letters denote significant differences between treatments; ANOVA, $P < 0.05$

2.5 Discussion

When presented with a choice, *A. lentisci* were not deterred from AR37-infected perennial ryegrass. Results suggest that *A. lentisci* do not initially (within 24 hours) perceive endophyte (AR37) and thus do not display a deterrent behavioural response. This is an interesting finding because AR37 is known to have a toxic effect on this aphid. Popay and Cox (2016) conducted a series of no-choice Petri dish experiments counting aphid populations on endophyte-infected (AR37, common-toxic and AR1) and endophyte-free perennial ryegrass plants (cultivar 'Grasslands Samson'). *Aploneura lentisci* grown on AR37 appeared healthy for 7 - 20 days before aphids developed tremors and died. Results suggested that AR37 did not have an anti-feedant effect but was toxic to *A. lentisci* when plant material was consumed. Tremors indicated the involvement of a neurotoxin and the delayed response suggested an inducible secondary metabolite or slow acting toxin. The present data combined with previous studies, suggests that in the field *A. lentisci* would be equally attracted to AR37-infected and endophyte-free ryegrass and are unlikely to be deterred by an anti-feedant compound. Instead aphids would feed and reproduce at a normal rate, at least for a short time, until they are adversely affected by the neurotoxin.

Herbivorous insects are known to possess mechanisms which enable them to detect and reject plants that contain harmful secondary compounds as ingestion of these compounds can result in death. An example is the fall armyworm which was shown to utilise a post-ingestive response mechanism to detect the toxic compound indole 3-carbinol. In experiments, caterpillars fed on diet containing this compound for up to 3 minutes before stopping and becoming motionless (Glendinning & Slansky, 1995; Glendinning, 2002). Initial biting activity of the caterpillar was not inhibited, indicating a post-ingestive rather than a pre-ingestive mechanism (Glendinning, 2002). Another example is *Menduca sexta* larvae which detect the toxic compound aristolochic acid, resulting in inhibition of feeding (Glendinning *et al.*, 1999). Experiments indicate a role of contact chemoreception in perception. Caterpillars that no longer contained selected chemosensilla ingested diet and this affected growth rates when compared to equivalent controls (Glendinning *et al.*, 2001). The interaction investigated in the present study is interesting as the source of the toxin is not the host plant itself but the endophyte. Endophyte-free ryegrass and ryegrass infected with the AR1 endophyte strain or the CT endophyte strain are all suitable host plants for *A. lentisci* (Popay *et al.*, 2004; Hume *et al.*, 2007; Popay & Gerard, 2007; Popay & Thom, 2009), although the CT strain has occasionally been found to have some negative effects on root aphid (Popay & Gerard, 2007). The chemical profile of AR37 is quite different to that of the AR1 and CT strains, which are also more closely related (Ball *et al.*, 1997a; Ball *et al.*, 1997b; Finch *et al.*, 2010; Johnson *et al.*, 2013). Although *A. lentisci* has been present in New Zealand pastoral ecosystems since at least the 1930s (Cottier, 1953), the endophyte strain AR37 is a relatively recent introduction (2007) and thus these species have not co-evolved in these habitats. Perhaps selection

pressure will result in *A. lentisci* developing a mechanism to initially detect and subsequently reject plants containing the AR37 endophyte over time.

Subterranean root-feeders are not as well studied as their above ground counterparts due to the inherent difficulties in studying these insects *in situ*. This is particularly true for *A. lentisci*, for which extensive research has been conducted on the above ground, gall-inducing life stage in the Mediterranean (Wool & Manheim, 1986, 1988; Wool & Sulami, 2001; Wool, 2005; Nahum *et al.*, 2010), but very little on the below ground stage on the roots of Poaceae (Popay & Cox, 2016). This can be attributed to the small size and fragility of these aphids, particularly the nymphs which were found to be virtually invisible in the soil, as well as their observed sensitivity to disturbance or change in abiotic conditions. Because of this *A. lentisci* are a challenging insect to study. Here I developed a below ground choice bioassay which was able to effectively monitor root aphid choice. Future studies should utilise this method to investigate host-selection of *A. lentisci* when aphids are presented with a choice between alternative secondary hosts such as Italian ryegrass (*Lolium multiflorum*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) as *A. lentisci* preference of secondary hosts is not understood.

In this study I aimed to gain a basic understanding of the host-searching behaviour of *A. lentisci* as determining what stimuli are important in the initial stage of host-searching may improve management strategies for the control of this pest in the future. I hypothesised that *A. lentisci* would rely on olfactory stimuli to locate new hosts and to test this hypothesis I developed a methodology for an olfactometer to investigate olfactory responses of apterous *A. lentisci* nymphs to their host plant. In these olfactometer experiments *A. lentisci* did not orientate themselves towards the roots or herbage of perennial ryegrass. This indicates that under the experimental conditions I used these aphids do not use olfaction to locate hosts. Despite this result, I cannot rule out the involvement of olfactory stimuli as host-searching behaviour can be complex and involve multiple complementary stimuli (Tuttle *et al.*, 1988). For example, visual stimuli in combination with olfactory cues can be important for some species (Chapman *et al.*, 1981; Todd *et al.*, 1990; Blackmer & Canas, 2005; Patt & Sétamou, 2014) such as the tea aphid (*Toxoptera aurantii*) which Han *et al.* (2012) found was more strongly attracted to light yellow and green sticky boards when these boards contained intact tea shoot volatiles (vial containing a shoot volatile solution) (*Camellia sinensis*) than without.

A. lentisci can be very sensitive and due to the lack of knowledge of the behaviour of this insect it is possible that my testing protocol was not ideal. For example, we do not know what time of the day this aphid searches for new hosts, whether dispersal is seasonal, what life stage is responsible for dispersal and whether aphids move through the soil or on the soil surface when searching for new hosts. In addition, season, temperature, diurnality and the intensity of solar radiation (Kirstine *et al.*, 1998; Holopainen & Gershenzon, 2010) can affect the volatile blend emitted from a plant. Differences

in the volatile blend may then affect an insect's detection and response to its host plant in an olfactometer experiment. Apterous and alatae morphs of the same species may also respond to volatile blends differently. Here I tested apterous *A. lentisci* rather than alatae morphs as the latter have never been observed when sampling below ground populations in New Zealand (Popay & Cox, 2016). Alatae aphids, however, are often considered to be primarily responsible for host-searching in other species as they have the ability to disperse over greater distances. It is possible that alatae virginoparae (an aphid that is produced by parthenogenesis) are produced at certain times of the year in New Zealand and are involved in dispersal of the clones between secondary host plants. Alatae *A. lentisci* have been recorded in the literature, in sticky traps in Canterbury, New Zealand in the 1960s (Lowe, 1968) and in colonies maintained in a climate chamber (Müller, 2019) but it is unclear whether these alatae were sexupare or alatae virginoparae. Blackman and Eastop (2000) reported that flights of alatae in New Zealand in the late summer were largely made up of sexupare. Sexupare are the life stage that form for dispersal from secondary hosts (Poaceae) back to primary hosts (*Pistacia lentiscus*). These aphids give birth to sexuales (males and female) which mate and produce an egg. When the fundatrix hatches from its egg it will induce a gall on *P. lentiscus*, a plant which is not found in New Zealand (see Müller, 2019). Whether alatae virginoparae are commonly produced on pastoral farms in New Zealand is not known (Müller, 2019).

Wool *et al.* (1994) observed migration of alatae sexuparae to the primary host, *P. lentisci*, in Israel. This showed that *A. lentisci* were able to distinguish between *Pistacia* and neighbouring tree species (pine, carob and almond). However, a number of individuals were trapped on other *Pistacia* species such as *Pistacia palaestina* and *Pistacia atlantica*. Although sexuales were produced on the wrong hosts, the fundatrices would have been unable to induce a gall and would not have survived. More landing errors were recorded for *A. lentisci* when compared with the other species studied. Wool (2005) suggested that galling aphids may have adopted a 'broadcasting' dispersal strategy where a large number of genetically identical offspring are released in the hope that some will land on the correct host species. However, some pre-contact cues must be involved as *A. lentisci* were not trapped on neighbouring trees of a different genus. In New Zealand, *A. lentisci* apterous aphids occur on the roots of Poaceae year-round, although they can also be found on the leaves of these plants (Rasmussen *et al.*, 2008). In this study I assumed that *A. lentisci*, like many other aphid species, actively select new hosts. An alternative hypothesis is that apterous nymphs disperse passively. This life stage is small and lightweight and may float in water (Salt *et al.*, 1996) or be blown with the wind (Blackman & Eastop, 2000), landing on a suitable host plant by chance. Further investigation of the morphological forms present in New Zealand is warranted. Intensive field trapping would establish whether alatae virginoparae are produced at certain times of the year and if nymphs are dispersed by wind.

The volatile compounds emitted by perennial ryegrass herbage and the roots of a *Lolium perenne* x *Festuca pratensis* hybrid have been identified and quantified (Hopkins & Young, 1990; Pańka *et al.*, 2013a; Qawasmeh *et al.*, 2015; Rostás *et al.*, 2015) and some compounds have been shown to elicit behavioural and electroantennogram responses in other aphids species (Visser & Piron, 1995; Han *et al.*, 2012). The more ubiquitous volatile compound, carbon dioxide, is also thought to play a role in host-searching behaviour of soil dwelling insects, although it is unlikely an organism could use this volatile alone to locate hosts (Johnson *et al.*, 2006; Johnson & Nielsen, 2012). CO_2 has a low molecular mass, can diffuse over long distances and has been shown to be attractive to some soil dwelling insects (Bernklau & Bjostad, 1998b). However, at very high concentrations, CO_2 can have a repellent or even toxic effect on insects (Bernklau & Bjostad, 1998a; Johnson & Nielsen, 2012). It is possible that an accumulation of CO_2 in my olfactometer, as a result of the still-air design, could have deterred aphids from the roots of their host plant. Future experiments could adapt my olfactometer design so that purified air is pushed through and extracted. This would also help to circulate volatile compounds which are produced in low concentrations by the roots.

Additional experiments are required to clarify initial host-searching behaviour of *A. lentisci*. Electroantennograph bioassays could be carried out to determine if the volatile blend or individual compounds activate receptor neurons located on *A. lentisci* antennae. Although these experiments would not provide information on what behavioural response may be triggered they would indicate whether *A. lentisci* can detect these compounds.

This study investigated aspects of the host-searching, selection and acceptance behaviour of apterous *Aploneura lentisci* to endophyte-infected and endophyte-free perennial ryegrass. The host preference assay suggests that *A. lentisci* are unable to initially perceive the potent endophyte strain, AR37, in perennial ryegrass host plants, demonstrating that negative effects of endophyte are not always associated with initial perception and avoidance behavioural responses. Furthermore, olfactory experiments indicate that under the experimental conditions I used olfaction does not appear to be an important factor influencing host-selection behaviour of highly mobile apterous nymphs which are thought to be involved in dispersal in New Zealand's intensive pastoral ecosystems. However, the role of olfactory stimuli cannot be completely ruled out from these experiments alone. While there has been considerable interest in understanding olfactory responses of above ground aphid species (Chapman *et al.*, 1981; Bernasconi *et al.*, 1998; Quiroz & Niemeyer, 1998; Webster *et al.*, 2008; Yang *et al.*, 2009; Han *et al.*, 2012), to the best of my knowledge, this is the first study to investigate olfactory responses of a below ground aphid species.

Chapter 3

Olfactory Responses of Argentine Stem Weevil (*Listronotus bonariensis*) to Endophyte-infected (*Epichloë festucae* var. *lolii*) and Herbivore Damaged Perennial Ryegrass (*Lolium perenne*)

3.1 Abstract

This study investigated the role of plant volatiles in endophyte-mediated defence of a major agricultural grass species (*Lolium perenne*). The asexual fungal endophyte (*Epichloë festucae* var. *lolii*) colonises perennial ryegrass in a defensive mutualistic interaction, which provides host plants with protection against phytophagous pest insects. Anti-feedant effects and the defensive properties of endophyte-derived alkaloids have been well documented, but few studies have investigated whether plant volatiles are involved in defence. Olfactometer bioassays were performed to evaluate behavioural responses of Argentine stem weevil (ASW, *Listronotus bonariensis*) to perennial ryegrass subject or not to conspecific herbivory and in the presence or absence of endophyte (AR1 or common-toxic endophyte). Results established that ASW adults are able to utilise olfaction to orient towards the volatiles released by perennial ryegrass and weevils displayed a preference for plants previously damaged by conspecific weevils. Interestingly, there was no evidence that ASW adults had the ability to distinguish between endophyte-infected (AR1 and common-toxic strains) and endophyte-free plants using olfaction alone. Using a push-pull extraction technique, thirteen volatile compounds were identified in the volatile blend released by perennial ryegrass and endophyte and herbivory were found to alter the volatile compounds and quantities emitted. This study suggests that despite observing differences in the plant volatile blend, ASW do not perceive the endophyte using olfaction alone and must rely on other cues, e.g. contact chemoreception or post-ingestional malaise, to detect the presence of a bioactive endophyte in an otherwise acceptable host.

3.2 Introduction

Endophytic fungal symbionts of the genus *Epichloë* co-evolved with grasses from the Poaceae family. Asexual morphs, which colonise hosts asymptomatically, have been studied intensively as they form defensive mutualistic associations with the major agricultural grass species *Lolium* and *Festuca* (Clay, 1988; Johnson *et al.*, 2013; Young *et al.*, 2013). Simultaneous discoveries in the early 1980s identified that the fungal endophyte strains (genotype) colonising perennial ryegrass (*Lolium perenne* Linnaeus, Poales: Poaceae) in New Zealand and tall fescue (*Festuca arundinacea*) in North America produced mycotoxins that were responsible for causing livestock toxicosis under certain grazing conditions (Bacon *et al.*, 1977; Fletcher & Harvey, 1981; Gallagher *et al.*, 1981; Schmidt *et al.*, 1982; Fletcher *et*

al., 1990). These endophytes are commonly referred to as common-toxic (CT) strains (formerly known as wild-type or standard endophyte). Removing the toxic endophyte strain was not an option in New Zealand's intensive pastoral ecosystems as plants were left highly susceptible to insect attack (Tapper & Latch, 1999). A survey of the chemical diversity among endophyte-infected ryegrass in New Zealand was undertaken in the 1980s with the aim of identifying an endophyte strain that provided the plant with insect resistance, but which did not produce the mycotoxin lolitrem B (Tapper & Latch, 1999). Despite analysing hundreds of plants, all were found to contain this compound so the search was extended to include diverse grassland in Europe (Tapper & Latch, 1999). Several strains that met this criterion were identified and have since been successfully commercialised and are sold to farmers in New Zealand, Australia, USA and South America within the seed of host plants (Latch & Christensen, 1985; Caradus *et al.*, 2013a; Johnson *et al.*, 2013; Johnson & Caradus, 2019). Newer pastures in New Zealand commonly contain one of the 'selected' endophyte strains (AR1 and AR37), but older pastures are still likely to be infected with the naturalised common-toxic (CT) strain. In reality, pure swards of endophyte-infected plants are unlikely as endophyte-infected seed is sensitive to storage conditions and uncertified seed is also planted (Rolston *et al.*, 1986; Hume & Barker, 2005; Hume *et al.*, 2013). Swards on-farm therefore contain a mixture of endophyte-free and endophyte-infected plants growing in close proximity.

Pastoral ecosystems in New Zealand are unique as many of the most destructive insect pests are non-indigenous and have arrived in New Zealand from different parts of the world. Phytophagous pest insects feed on both the above and below ground plant structures and can destroy significant areas of pasture if left uncontrolled as there are few natural predators to control populations (Johnson *et al.*, 2013; Ferguson *et al.*, 2018). The Argentine stem weevil (ASW, *Listronotus bonariensis* (Kuschel, 1955), Coleoptera: Curculionidae) was accidentally introduced to New Zealand in the early 20th century from South America (Williams *et al.*, 1994) and has since become a key economic pest of perennial ryegrass (Prestidge *et al.*, 1991; Ferguson *et al.*, 2018). Highly mobile adults deposit their eggs in the pseudostems of chosen hosts which enables destructive stem-boring larvae to mine the centre of the plant. Adults feed on the leaves of tillers, creating distinctive window-like feeding scars and may sever ryegrass seedlings by feeding on the basal region. Biological control of this species in New Zealand involves fungal endophytes and a parasitic wasp, *Microctonus hyperodae*, which parasitizes adult weevils resulting in sterilization and eventual death (Barker *et al.*, 1984a; Barker *et al.*, 1984b; Goldson *et al.*, 1994; Goldson *et al.*, 1998b; Popay *et al.*, 1999; Thom *et al.*, 2013; Ferguson *et al.*, 2018). Parasitoids have been known to influence the behaviour of their host (Weinersmith, 2019) and this will be considered in this study.

Both the naturalised CT and 'selected' AR1 endophyte strains reduce ASW adult feeding and deterrent effects have primarily been attributed to the endophyte-derived metabolite peramine (Barker *et al.*,

1984b; Rowan & Gaynor, 1986; Popay *et al.*, 1990; Popay *et al.*, 1999; Popay & Thom, 2009). Antifeedant effects were confirmed when this compound was incorporated into a semi-synthetic diet experiment (Popay *et al.*, 1990), but the mode of action has not been documented.

In addition to endophyte-derived alkaloids, recent studies have begun to investigate whether volatile organic compounds are involved in plant defence. All plants release diverse volatile bouquets which can enable interactions with other organisms in their environment. Volatiles can have direct defensive functions, or they can act indirectly by attracting parasitoids or predators of the attacking herbivorous insect (Allmann & Baldwin, 2010; Kappers *et al.*, 2011). Plants alter their volatile profile in response to changes in their abiotic or biotic environment, including in response to herbivory and when infected with an endophyte (Yue *et al.*, 2001; Jallow *et al.*, 2008).

Herbivorous insects have evolved highly sophisticated olfactory systems than enable them to detect and exploit difference between plant volatile profiles which they use as cues to orient towards suitable hosts for feeding and oviposition (Visser, 1986; Quiroz & Niemeyer, 1998; Bruce *et al.*, 2005; Szendrei *et al.*, 2009; Branco *et al.*, 2019). Host-searching herbivores can, in some cases, use the volatile blend emitted by a plant after it is attacked by conspecific or heterospecific insects (herbivore induced plant volatiles, HIPVs) to help locate plants or avoid unsuitable hosts (De Moraes *et al.*, 2001; Szendrei *et al.*, 2009; Magalhães *et al.*, 2012; Ogah *et al.*, 2017). In an unpublished study, ASW adults were shown to orient towards damaged Italian ryegrass in an olfactometer (J. Vereijssen, personal communication), which suggests that ASW may utilise HIPVs to help locate favourable hosts. Whether a similar response is observed in response to perennial ryegrass or endophyte-infected grasses is unknown.

Qawasmeh *et al.* (2015) reported differences in the volatile blend emitted by endophyte-infected and endophyte-free perennial ryegrass. Bioassays indicated that African black beetle adults (*Heteronychus arator*, Coleoptera: Scarabaeidae) in Australia avoid the volatile blend emitted by perennial ryegrass infected with the AR1 or CT, but not the AR37 endophyte strain. This was an interesting finding given that AR1 is only weakly deterrent to ABB, whereas AR37 provides ryegrass with a strong level of protection (Popay & Baltus, 2001; Popay & Thom, 2009). In addition, Rostás *et al.* (2015) found that root feeding larvae of the native New Zealand grass grub (*Costelytra giveni* formerly *Costelytra zealandica*, Coleoptera: Scarabaeidae) were able to exploit differences in the volatile blend to avoid an endophyte-infected (*Epichloë uncinata* formerly *Neotyphodium uncinatum*) hybrid grass (*Festuca pratensis* x *Lolium perenne* cultivar GrubOUT®). This suggests that *Epichloë* endophytes, which colonise above ground tissues, are also capable of altering the volatile blend emitted by roots and thus altering the distribution and feeding habits of below ground herbivores.

The purpose of this chapter was to investigate the role of plant volatiles in orientation and selection behaviour of ASW adults in response to endophyte (AR1 and CT) and herbivory. Still-air olfactometer

experiments were used to examine whether ASW use olfaction to first locate host plants and then to select endophyte-free in preference over endophyte-infected ryegrass plants. ASW olfactory responses to herbivore damaged plants were also investigated as was the combined effects of endophyte and herbivory. Volatiles emitted by damaged and undamaged, endophyte-free and AR1-infected ryegrass were collected and analysed, to explore possible differences existing between the volatile profiles emitted by different treatments.

3.3 Methods

3.3.1 Establishment of ryegrass plants and endophyte testing

Endophyte-infected (AR1 and CT) and endophyte-free perennial ryegrass plants (*Lolium perenne* cultivar 'Grasslands Samson') were established from seed obtained from the Margot Forde Germplasm Centre, AgResearch (Palmerston North, New Zealand). Seeds were germinated in Petri dishes (90 mm) lined with damp filter paper (1 mL tap water) and held inside a darkened container at 20°C for 7 - 10 days. Germinated seedlings were planted into individual, identifiable positions in polystyrene planter boxes filled with fresh potting mix (Daltons™). Plants were maintained with regular hand watering and trimming.

All plants were tested for endophyte-infection using a tissue print immunoassay at least 6 weeks post germination. Tillers (1 - 2 per plant) were cut from the base of the plant, where endophyte mycelium is concentrated and dead sheath material and soil removed. The cut surface was then pressed firmly onto a piece of nitrocellulose paper. 'Blots' were developed using an immunoassay described by Simpson *et al.* (2012), although incubation and washing methods were modified at AgResearch Ruakura to improve endophyte detection (see 2.3.1). Only plants of the correct endophyte-infection status were kept for experiments.

The size of each plant was reduced 5 - 6 days prior to each bioassay (in Experiment 2 date 1 this occurred three days prior due to mortality caused by a malfunction in the glasshouse facilities) by removing additional tillers so that all plants had approximately 8 tillers. Plants were re-potted into individual plastic specimen contains (75 mL) which were placed into boxes filled with damp sand and maintained in a glasshouse until required.

3.3.2 Argentine stem weevil (*Listronotus bonariensis*)

Field collected Argentine stem weevil adults were chosen for this study as it is important to understand how naturally occurring, diverse populations respond to these compounds. Weevils were collected from pastures (Ruakura Research Centre, Hamilton, New Zealand) using a reverse modified blower vacuum no more than 48 hours before the beginning of each experiment (24th January and 8th March

2017 [summer to early autumn]). Weevils were then sexed using the external morphological features described by Goldson and Emberson (1981). ASW were removed from the litter in the collection and placed into a -20°C freezer for 6 minutes. This briefly reduced activity and allowed examination of the thorax under a stereomicroscope. Female weevils were chosen for experiments as sex-specific responses have been documented in the literature with females often showing stronger responses to plant volatiles than males (Szendrei & Rodriguez-Saona, 2010; McGraw *et al.*, 2011). All weevils were starved for 24 hours at room temperature prior to inclusion in an olfactometer experiment.

Following the completion of each experiment the sex of each ASW was confirmed by dissection. ASW were secured for dissection in a plastic Petri dish (90 mm) that was half filled with carbon-blackened paraffin wax. The ventral surface of each weevil was pressed into molten wax and a layer of tap water added to the surface to aid in rehydration and suspension of internal organs. The elytra and wings were removed and the dorsal surface of the abdomen peeled back to expose the gut and gonads (Figure 3.1A). Due to the difficulties in sexing ASW using external features a small number of males were identified as having been included in experiments.

In addition to determining sex, the presence or absence of parasitoid (*Microctonus hyperodae*) larvae (Figure 3.1B) was noted (Goldson & Emberson, 1981). Although it is possible to purge parasitoids from a population of field collected weevils, this was not done as parasitism rates were expected to be low and rearing insects in a colony could have influenced their behaviour.

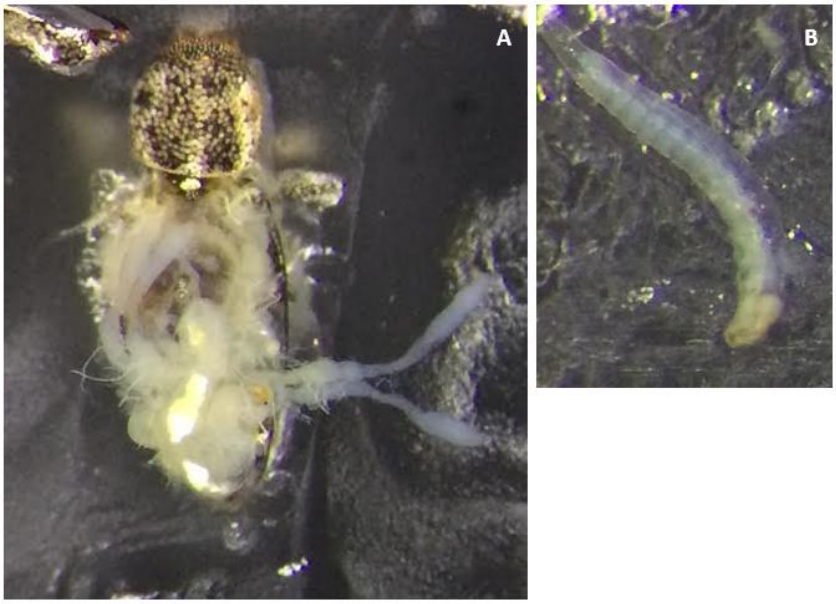


Figure 3.1: **A)** Dissected Argentine stem weevil adult (*Listronotus bonariensis*) showing the female reproductive system. **B)** a *Microctonus hyperodae* larva removed from an adult weevil.

3.3.3 Olfactometer

A glass still-air olfactometer was modified from Van Tol *et al.* (2002) and the experimental protocol adapted to ASW behavioural and morphological characteristics (Figure 3.2). The olfactometer design allowed ASW adults to select between two odour sources or remain in the central arena. The olfactometer consisted of a large glass Petri dish (145 mm diameter (excluding thickness of glass) with two small circular openings (13 mm diameter) on the bottom section of the dish (82 mm apart and 16 mm from the rim). Attached to each opening was a short tube (26 mm length) of equal diameter (13 mm) to the opening which led into larger cylindrical holding tubes (69 mm length x 44 mm diameter). Below the two holding tubes and separated by a fine mesh barrier (100 mm x 100 mm), were glass cups (73 mm length x 44 mm diameter (52 mm diameter at the top rim)) that held whole plants. The Petri dish became the 'test arena' where 10 ASW adults were placed at the beginning of each experiment. Weevils' choices were recorded based on the number of weevils found in different cylindrical holding tubes.

Figure 3.2: Still-air olfactometers in a controlled environment chamber.

3.3.4 Olfactory experiments

Olfactory response to host plants and endophyte

Three olfactometer experiments were carried out to investigate the role of olfaction in selection of endophyte-free and endophyte-infected (AR1 and CT endophyte) plants. In Experiment 1 ASW were presented with a choice between the volatile blend released by their host, endophyte-free ryegrass, and a control of damp cotton wool in still-air olfactometers (9 replicates were run in parallel). In Experiment 2 weevils were offered a choice between AR1-infected and endophyte-free ryegrass (29

replicate olfactometers; 10 run in parallel on date 1 and 19 run in parallel on date 2) and between CT-infected and endophyte-free ryegrass in Experiment 3 (19 replicates run in parallel).

Each plant was removed from its container, the roots placed into a plastic bag and the whole plant folded into a glass cup. A clean square of mesh was positioned over the top of each cup and the olfactometer constructed. Dry cotton wool was placed around the edges of the Petri dish and Teflon thread seal tape (brand Plumb it) was used to cover small gaps between joints. In Experiment 1, a ryegrass plant was placed in one cup and damp cotton wool was added to the second cup to act as a control. Humidity was measured in three of the replicates that contained plant material. This showed that 500 mg of cotton wool plus 2 mL of Milli-Q water was sufficient to create a similar humidity in control cups.

Olfactometers were placed into a controlled environment chamber (20°C, 80% humidity, no light) for 2 hours to allow diffusion of plant volatiles. Ten weevils that had been starved for 24 hours were added to the test arena and left to select an odour source overnight (15 - 16 hours), a time when weevils are most active during the summer (Barker & Pottinger, 1986). In the morning, olfactometers were deconstructed and the position of each weevil recorded (test arena, treatment or control). Weevils were frozen for later dissection. Each olfactometer was rinsed with warm tap water and then purified water before it was wiped clean with petroleum spirit and acetone and left to bench dry. To prevent contamination, new mesh squares were made for each experiment. Plant position in each olfactometer was randomly orientated between the two possible positions.

Olfactory response to herbivory and combined effects

Three olfactometer experiments (Experiments 4 - 6) were carried out to investigate ASW olfactory response to damaged (conspecific insects) endophyte-free and endophyte-infected plants. In Experiment 4, ASW were presented with a choice between undamaged and damaged endophyte-free ryegrass (18 replicate olfactometers; 9 run in parallel on date 1 and 9 on date 2). This bioassay was repeated in Experiment 5 using AR1-infected ryegrass plants (19 replicate olfactometers; 9 run in parallel on date 1 and 10 on date 2). In the final experiment weevils were presented with damaged endophyte-free and damaged AR1-infected plants (20 replicates).

ASW that were used to damage experimental plants were collected fresh from the field, sorted into plastic specimen containers and starved for 24 hours. Forty-eight hours before the beginning of an olfactometer experiment, all plants (including those that were to remain undamaged) were placed individually into plastic takeaway containers (170 mm x 120 mm x 70 mm) of which one side of the container consisted of a fine mesh (Figure 3.3). Containers were placed into a glasshouse and plants were watered as required. In Experiment 4, five weevils were caged onto each endophyte-free plant and in Experiment 5 seven weevils were caged on to each AR1-infected plant. Weevil numbers were

increased in Experiment 5 to ensure sufficient feeding damage to endophyte-infected plants. Experiment 6 contained both AR1 and endophyte-free plants and five weevils were caged onto each plant. Weevils were removed from plants immediately before the bioassay was performed.

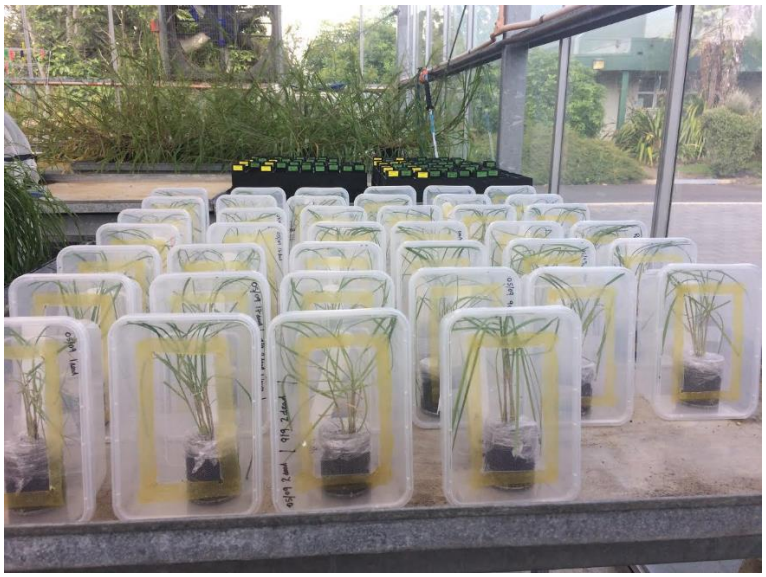


Figure 3.3: Argentine stem weevil (*Listronotus bonariensis*) adults caged onto individual ryegrass (*Lolium perenne*) plants in a glasshouse.

3.3.5 Collection and analysis of volatile organic compounds

Emissions were collected from ryegrass plants in June 2017 (winter). A push-pull system was used for dynamic headspace sampling of the volatiles emitted by perennial ryegrass (Figure 3.4). Volatile compounds were collected from undamaged endophyte-free, damaged endophyte-free, undamaged AR1-infected and damaged AR1-infected perennial ryegrass plants. For each plant-endophyte combination two 12-week-old plants were re-planted into a single specimen container (150 mL) and placed into a glass collection vessel. Volatiles were collected separately from 5 replicate plant pairings of the same treatment simultaneously. To account for contamination in the system a collection was made from vessels that contained empty specimen containers only. A compressed air cylinder was used to push charcoal-filtered air into each vessel at a rate of 0.8 L / min. Air was pulled through a SuperQ absorbent filter (30mg ARS Inc., Gainesville, FL, USA), at the same rate, using a vacuum pump (ILMVAC GmbH, Germany). Volatiles were collected for 4 hours. Compounds were removed from the SuperQ filter with methylene chloride (150 µL) and 200 ng of tetralin (Sigma-Aldrich, Australia) was then added as an internal standard to each sample. Samples were run through a gas chromatograph coupled with a mass spectrometer (Shimadzu GC-MS-QP2010 Ultra) which was equipped with a Restek Rtx-5ms fused silica capillary column (30.0 m x 0.25 mm i.d. x 0.25 µm, Bellefonte, PA, USA) by Jason Breitmeyer. Samples (1.5 µL) were injected in pulsed splitless mode (241 kPa pulse for 39 seconds) at

220°C. Initial oven temperature was set at 35°C held for 3 minutes then increased at 8°C / min to 320°C for 8 min. The carrier gas was helium (1.75 mL/ min). Volatile compounds were analysed using GC-MS solution version 4.11 and were tentatively identified by comparing their mass spectra with entries in the NIST 11. In addition, experimental retention indices were compared to those listed on the National Institute of Standard and Technology (NIST) Webbook (https://webbook.nist.gov/chemistry/). Volatiles were quantified by comparing the area under each peak to that of the internal standard.

Figure 3.4: Dynamic headspace sampling of the volatile organic compounds emitted by perennial ryegrass (*Lolium perenne*).

3.3.6 Statistical analyses

A multinomial regression analysis was performed on weevil position data (i.e. number of weevils from a replicate in the test arena, treatment and control position) in each olfactometer experiment. The effect of parasitism (a fixed factor with two levels) was analysed using a linear mixed model analysis fitted by a residual maximum likelihood (REML). The random model was comprised of the nuisance factors; replicate, orientation (two levels; left and right), date (where applicable), sex (only applied to Experiment 3) and two factors relating to the position of the olfactometer in the controlled environment chamber; shelf (two levels - bottom or top) and side (two levels but this was omitted for Experiments 4 and 5 as aliased with date). Replicate was the only nuisance factor included in the analysis of data from Experiment 1, due to lower replication. All random effects were constrained to be positive. The statistical significance of the fixed effects was assessed using approximate F-tests at the 5% significance level. In Experiment 2 the fixed effect in the model included the additive effects of sex as 19 males were mistakenly included in the experiment.

Assessments of ASW adult feeding damage were made by counting the number of window-like feeding scars on all leaves. An analysis of variance (ANOVA) was performed on feeding scar data in Experiments

4, 5 and 6. Statistical significance was determined using Fisher's unprotected least significant difference posthoc test conducted at the 5% significance level.

A principal component analysis (PCA) was performed on volatile emission data. The PCA analysis was based on a correlation matrix. Replicate 1 of the treatment 'AR1-infected undamaged' was removed from the analysis due to some missing values. A general or unbalanced (as appropriate) one-way ANOVA blocked by replicate was performed on emission data for each of the compounds separately. A natural transformation (log10) was necessary for some of the compounds. In data sets which included zero values, half the value of the smallest concentrations was added to the data prior to analysis. For some treatments just one of the five replicates contained a value above zero and these treatments could not be included in the ANOVA. Statistical significance was determined using Fisher's unprotected least significant difference posthoc test conducted at the 5% significance level.

All statistical analyses were conducted in Genstat 18th edition.

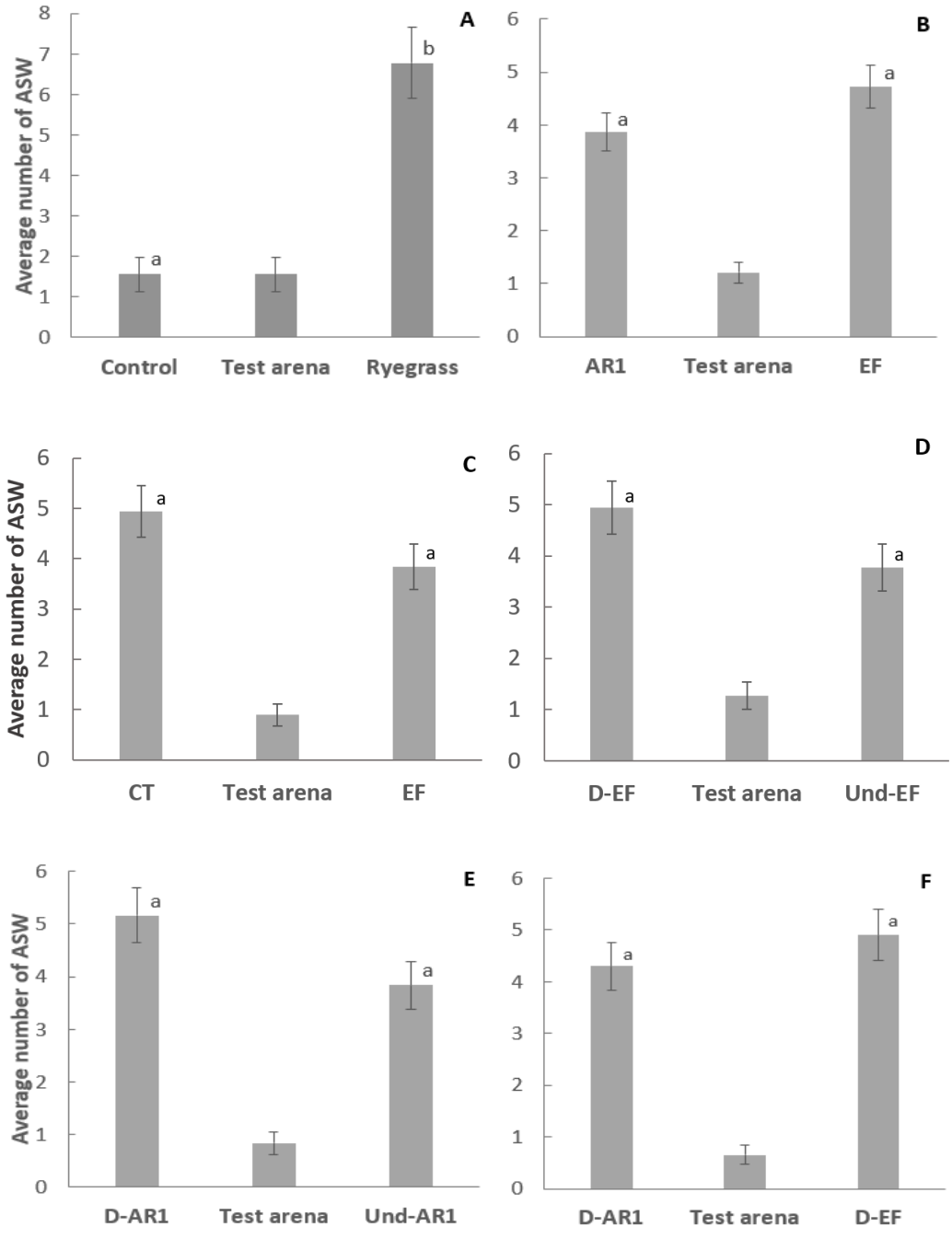
3.4 Results

3.4.1 Olfactory experiments

Six olfactometer experiments were run between 25th January and 10th March 2017. The number of weevils that moved from the test arena and selected one of the treatments was high, with response rates of 84 to 93% in all experiments.

Olfactory response to host plants and endophyte

In Experiment 1, ASW adults were significantly ($P < 0.001$, $t = 4.97$, r.d.f. [residual degrees of freedom]= 16, figure 3.5A) attracted to the volatile blend released by perennial ryegrass (endophyte-free), when given a choice between their host plant and a control of damp cotton wool. The effect of endophyte was then investigated by presenting weevils with a choice between the volatile blend emitted by endophyte-free and either AR1 (Experiment 2, Figure 3.5B) or CT (Experiment 3, Figure 3.5C) endophyte-infected perennial ryegrass plants. No significant differences in selection were found in response to either endophyte strain (Experiment 2, $P = 0.114$, $t = 1.58$, r.d.f. = 56 and Experiment 3, $P = 0.105$, $t = 1.62$, r.d.f. = 36).


A
Average number of ASW
Control
Test arena
Ryegrass
B
AR1
Test arena
EF
C
CT
Test arena
EF
D
D-EF
Test arena
Und-EF
E
D-AR1
Test arena
Und-AR1
F
D-AR1
Test arena
D-EF

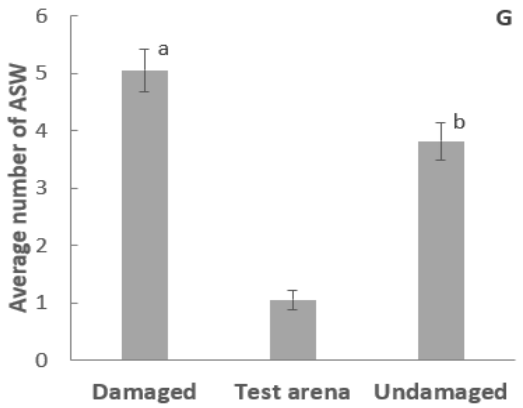


Figure 3.5: Response of Argentine stem weevil adults (ASW, *Listronotus bonariensis*) in still-air olfactometers to the volatiles released by perennial ryegrass (*Lolium perenne*). ASW were presented with a choice between **A** endophyte-free perennial ryegrass or a control of damp cotton wool (Experiment 1, n = 9); **B** AR1-infected ryegrass (AR1) and endophyte-free (EF) ryegrass (Experiment 2, n = 29); **C** common-toxic (CT)-infected ryegrass and endophyte-free (EF) ryegrass (Experiment 3, n = 19); **D** Undamaged endophyte-free ryegrass (Und-EF) and endophyte-free ryegrass previously damaged (D-EF) by conspecific insects (Experiment 4, n = 18); **E** damaged AR1-infected (D-AR1) ryegrass and undamaged AR1-infected (Und-AR1) ryegrass (Experiment 5, n = 19); **F** damaged AR1-infected (D-AR1) ryegrass and damaged endophyte-free (D-EF) ryegrass (Experiment 6, n = 20); **G** combined data from damaged (combined AR1 and EF) and undamaged (combined AR1 and EF) ryegrass. Bars represent the average number of weevils found in each chamber of the still-air olfactometer (± s.e.m [standard error mean]). Different letters above bars indicate significant differences; multinomial regression analysis, $P < 0.05$.

Olfactory response to herbivory and combined effects

More weevils selected damaged plants when presented with a choice between the volatile blend emitted by damaged and undamaged endophyte-free ryegrass plants (Experiment 4, Figure 3.5D), but this difference was not statistically significant ($P = 0.095$, $t = 1.67$, r.d.f. = 34). The combined effects of endophyte and herbivory were assessed by presenting weevils with a choice between damaged and undamaged AR1-infected ryegrass plants (Experiment 5, Figure 3.5E). In this experiment an average of 5.2 (± 0.52 (± s.e.m)) weevils selected the damaged plants, while 3.8 (± 0.45) weevils chose undamaged ryegrass ($P = 0.057$, $t = 1.91$, r.d.f. = 36). Because there was no effect of endophyte on selection the results from Experiments 4 and 5 were combined and analysed. More weevils selected damaged plants (Figure 3.5G) and this difference was significant ($P = 0.011$, $t = 2.53$, r.d.f 72). The total number of feeding scars on each damaged plant in Experiment 4 and 5 were assessed after the experiment. On average 29 (range 1 – 78) feeding scars were found on AR1-infected plants and 58 (range 13 – 102) on

endophyte-free plants. Seven AR1-infected plants had fewer than 15 feeding scars compared to just one endophyte-free plant. Damaged endophyte-free plants from Experiment 4 had significantly ($P < 0.001$, r.d.f = 17) more feeding scars than damaged AR1-infected plants from Experiment 5.

In the final experiment, ASW were presented with a choice between damaged endophyte-free and damaged AR1-infected plants. The average number of ASW that selected each plant type was similar and no significant difference in selection was identified ($P = 0.377$, $t = 0.88$, r.d.f. = 38) (Experiment 6, Figure 3.5F). When the number of feeding scars on these plants were assessed, endophyte-free plants were found to have significantly more ($P < 0.001$, r.d.f = 19) damage with an average of 103 (range 31 – 207) scars per plant compared to an average of 49 (range 5 – 87) on AR1-infected plants.

Effect of parasitism and sex

Parasitism rates and sex were confirmed at the conclusion of each experiment by dissection. Four weevils were not successfully dissected and were not included in the data analysis. On rare occasions (10 weevils) only sex and not parasitism could be determined due to degradation of the sample. Parasitism was low (between 11.9 and 23%) in all experiments except for Experiment 2 (EF vs AR1), in which a slightly higher number of parasitized weevils were found (32%). There was evidence for a significant effect of parasitism in Experiment 3 (EF vs CT), when 61% of not parasitized weevils were found to select the CT-infected ryegrass plants compared to 35% of the parasitized weevils ($P = 0.007$, $F_{1,158} = 7.37$, s.e.d. = 0.096). No more than two males were found in Experiments 1 (1.1%), 4 (1.1%), 5 (0.5%) and 6 (0.5%) but eight males (4.4%) were found in Experiment 3 (EF vs CT) and 19 (6.7%) in Experiment 2 (EF vs AR1). In Experiment 2 46% of females selected AR1-infected plants compared to 23% of males ($P = 0.064$, $F_{1,244} = 3.45$, s.e.d. = 0.1262). Interestingly, more males selected endophyte-free (n = 13) over AR1-infected (n = 3) host plants (3 weevils did not select a plant).

3.4.2 Volatile organic compounds

Herbage volatiles were collected using dynamic headspace sampling and analysed using GC-MS. Thirteen compounds were found and 9 were tentatively identified. Quantitative and qualitative differences were found between treatments (Table 3.1). There was an average of 126 (range 106 – 157) feeding scars on endophyte-free plants compared to an average of 52 (range 45 to 57) scars on AR1-infected plants.

Table 3.1: Volatile compounds emitted (ng/g fresh weight/h) by damaged and undamaged AR1-infected and endophyte-free perennial ryegrass (*Lolium perenne*) plants. RT = retention time, RI(e) = experimentally determined retention index, RI(d) = retention index from data bank (NIST WebBook), ND = not detected, * = found in 1 replicate only, NQ = not quantifiable due to co-elution with a contaminant.

				AR1 Damaged		AR1 Undamaged		EF Damaged		EF Undamaged	
Compound	**RT**	**RI(e)**	**RI(d)**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
3-Hexen-1-ol, acetate (E) or (Z)	10.20	1009	1005	3.96	0.97 - 5.16	4.19	1.35 - 5.76	1.10	0.30 - 3.08	1.92	0.71 - 40.58
Cis-β-ocimene	10.84	1040	1040	5.77	2.77 - 19.48	1.51	0.92 - 6.04	11.90	4.06 - 26.49	1.43	1.04 - 3.26
Trans-β-ocimene	11.06	1050	1052	3.17	1.79 - 14.93	1.21	1.00 - 2.94	7.85	2.53 - 13.73	1.91	1.39 - 2.32
Linalool	12.15	1102	1100	0.21	0.18 - 0.48	0.23	0.19 - 0.26	0.28	0.22 - 0.82	0.26	0.20 - 0.27
3,4-dimethylcyclohexanol	12.37	1113	1126	0.57	0.33 - 0.79	0.15	0.13 - 0.38	0.31	0.22 - 0.43	0.33	0.21 - 0.94
Indole	15.95	1306	1295	0.27	ND - 5.00	ND		1.24	0.05 - 9.50	0.91*	
Unknown (204, 119, 105)[a]	16.15	1318		0.25	ND - 7.20	2.91	0.91 - 7.26	0.31*		0.25*	
Unknown (95, 83)[a]	16.69	1351		0.14	0.13 - 0.21	0.08	0.05 - 0.09	0.18	0.12 - 0.24	0.22	0.07 - 0.27
Unknown (204, 91, 163)[a]	16.87	1361		NQ*		NQ		ND		ND	
Unknown (125, 125, 83)[a]	17.59	1404		NQ		NQ		NQ		NQ	
Dihydroactinolide	19.89	1551	1538	0.48	0.37 - 1.20	0.33	0.11 - 0.50	0.37	0.31 - 0.89	0.50	0.30 - 0.80
Neophytadiene	23.90	1840	1840	1.74	1.10 - 2.56	0.68	0.34 - 0.79	1.12	0.87 - 2.00	1.08	0.47 - 1.26
2-Pentadecanone,6,10,14-trimethyl	24.00	1847	1847	1.28	1.05 - 2.45	0.95	0.30 - 0.98	0.82	0.60 - 1.19	1.57	0.63 - 2.03

[a]The molecular ion (not included for unknown at 16.69), largest and second largest ion fragments are presented in brackets next to each unknown compound, respectively.

Effect of endophyte and herbivory on volatile compounds

A principal component analysis (PCA) was performed on emission data of the 11 compounds that could be quantified. Principal components 1 and 2 explained 59.36% of the variation (Figure 3.6). In the PCA, replicates of the same treatment tended to cluster together and this was particularly evident for replicates in the AR1-infected undamaged treatment. The compounds that were highly positively correlated (r = +0.80) were trans-β-ocimene and cis-β-ocimene, indole and trans-β-ocimene, as well as indole and cis-β-ocimene.

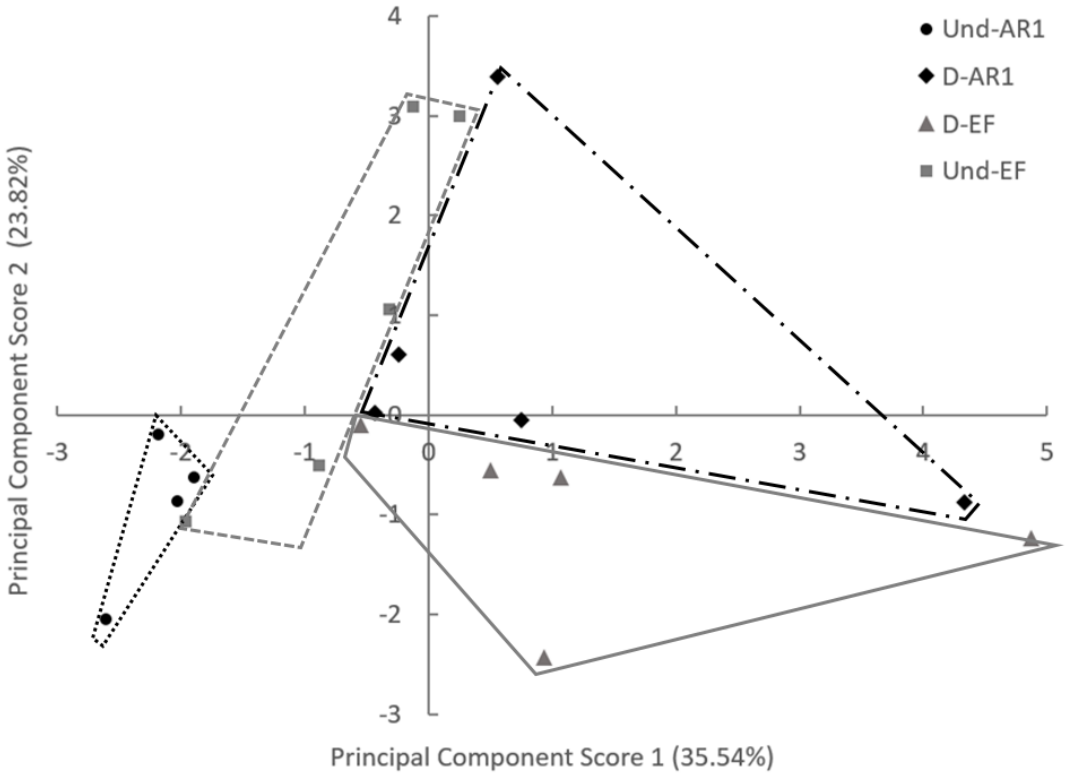


Figure 3.6: Scatterplot of principal component score 1 and 2 for volatile emission data. AR1-infected undamaged (Und-AR1), AR1-infected damaged (D-AR1), endophyte-free damaged (D-EF) and endophyte-free undamaged (Und-EF) perennial ryegrass (*Lolium perenne*). Plants were damaged by feeding caused by Argentine stem weevil adults (*Listronotus bonariensis*). Replicate 1 of AR1-infected undamaged plants was removed due to missing values. Geometric figures group the replicates of each treatment.

Endophyte significantly ($P < 0.05$) affected the emission of the unknown compound found at RT 16.69 with undamaged endophyte-free plants emitting a higher amount of the compound than undamaged AR1-infected plants. Emissions of a second unknown compound found at RT 16.15 were higher in AR1 undamaged plants than endophyte-free plants, where this compound was only found in one replicate.

The average emission rate of several other compounds varied between endophyte-free and AR1-infected plants (undamaged), but these differences were not found to be significant (note that the median value of the raw data is presented in Table 3.1 and mean values generated in GenStat are presented in Figure 3.7). An unknown compound which occurs at RT 16.87 could not be accurately quantified as it eluted closely with a contaminant, but interestingly this compound was only detected in AR1-infected plants and not endophyte-free plants. This unknown volatile has a molecular ion of 204, suggesting that this compound, and the unknown compound found at RT 16.15, could be sesquiterpenes.

Significant differences were also found between the volatile profile of damaged and undamaged plants. When looking at AR1-infected plants, emission rates were significantly different ($P < 0.05$) for six compounds; cis-β-ocimene, trans-β-ocimene, 3,4-dimethylcyclohexanol, unknown compound RT 16.69, neophytadiene and 2-pentadecannone,6,10,14-trimethyl (Figure 3.7). In these cases damaged plants emitted a greater amount of the compound than equivalent undamaged plants. Although the median concentration of the unknown compound found at RT 16.15 appears to be higher in undamaged plants in Table 3.1, this difference was not statistically different. Fewer significant differences were found between damaged and undamaged endophyte-free plants. Here the emission of just cis-β-ocimene and trans-β-ocimene were significantly ($P < 0.05$) higher in damaged plants. One qualitative difference was found with the compound indole (Figure 3.8) being emitted by both AR1-infected and endophyte-free damaged plants, but not equivalent undamaged plants with the exception of one replicate of an endophyte-free undamaged plant.

When investigating the combined effects of endophyte and damage (comparing AR1 and endophyte-free damaged plants) the median emission rate of cis-β-ocimene, trans-β-ocimene and indole were lower in AR1-infected plants, but differences were not found to be significant ($P > 0.05$). In contrast the emission rate of 3-hexen-1-ol acetate and 2-pentadecanone,6,10,14-tryimethyl were higher in AR1-infected plants but this difference was only significant ($P < 0.05$) for the latter compound.

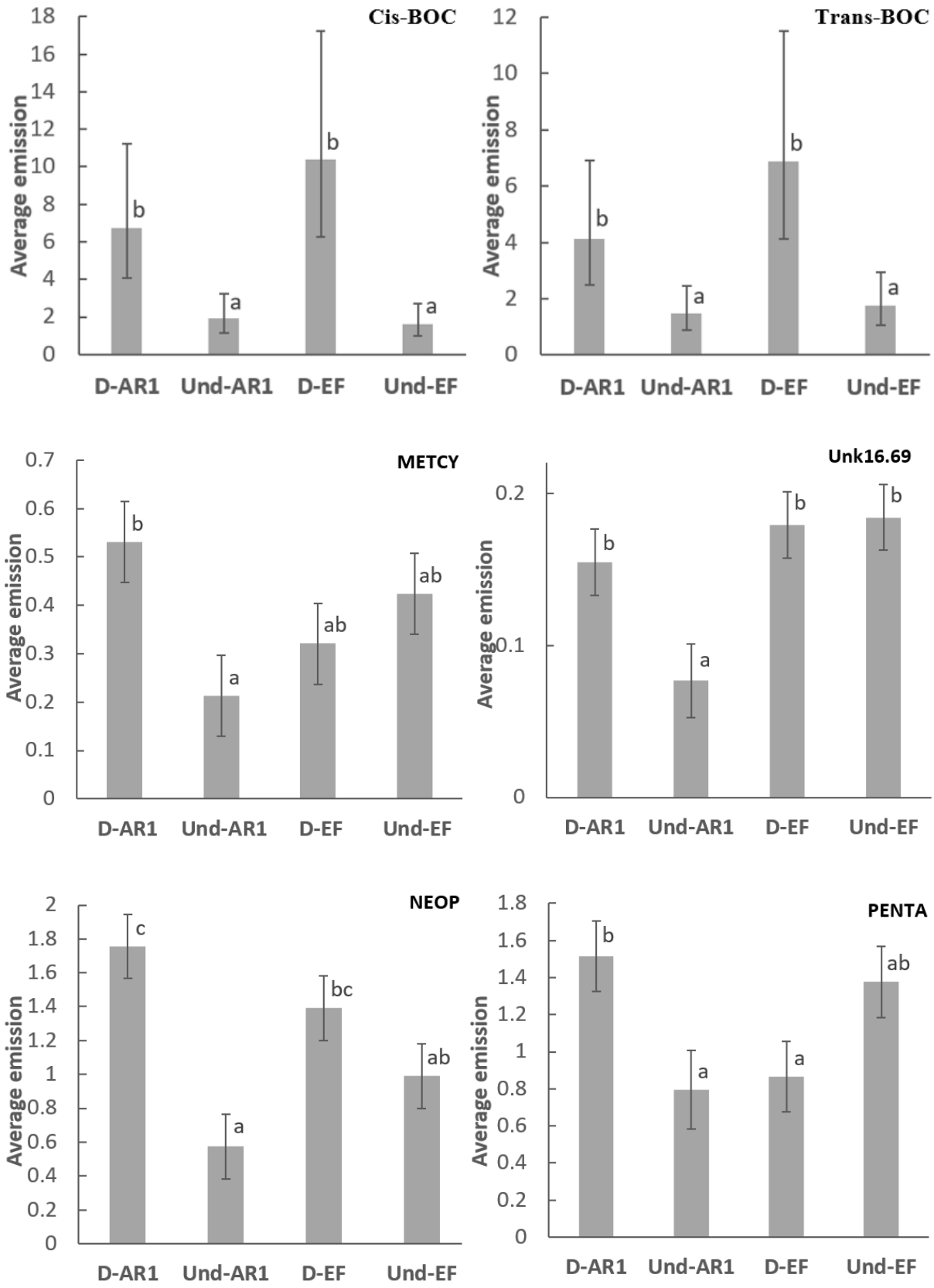


Figure 3.7: Average emission (ng/g fresh weight/h) rates of the five compounds that differed significantly between the four treatments; damaged AR1-infected (D-AR1), undamaged AR1-infected (Und-AR1), damaged endophyte-free (D-EF), undamaged endophyte-free (Und-EF) perennial ryegrass (*Lolium perenne*). Where required averages and 95% confidence intervals (C.I.) are back-transformed from the log scale. Compounds presented are; cis-β-ocimene (Cis-BOC) (± 95% C.I., ANOVA of log

transformed data), trans-β-ocimene (Trans-BOC) (± 95% C.I., ANOVA of log transformed data), 3,4-dimethylcyclohexanol (METCY) (± s.e.m. [standard error of the mean], ANOVA of untransformed data), unknown compound found at retention time 16.69 (Unk 16.69, ± s.e.m, unbalanced ANOVA of untransformed data), neophytadiene (NEOP) (± s.e.m., ANOVA of untransformed data) and 2-Pentadecanone, 6, 10, 14-trimethyl (PENTA) (± s.e.m, unbalanced ANOVA of untransformed data). Different letters above bars denote significant differences between treatments; analysis of variance, Fisher's unprotected least significant difference posthoc test conducted at the 5% significance level.

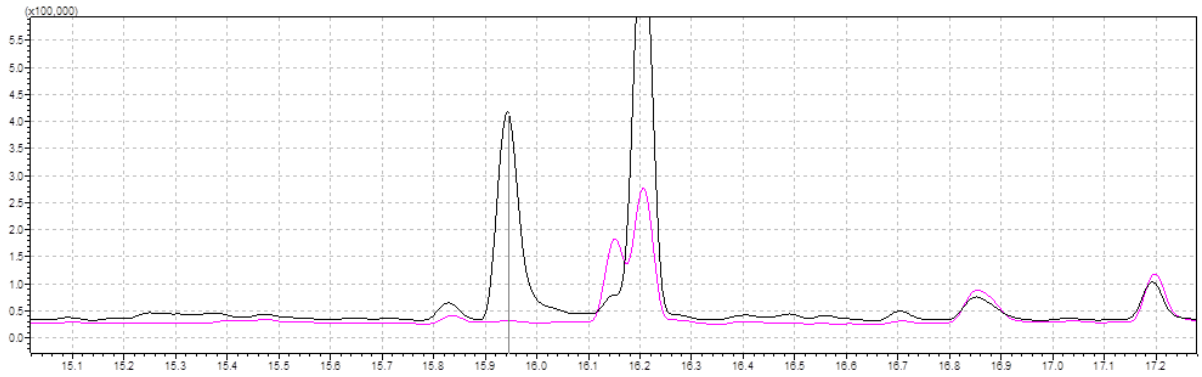


Figure 3.8: Chromatogram indicating indole (black vertical line through the peak, between RT 15.9 and 16) emitted by damaged AR1-infected perennial ryegrass (*Lolium perenne*) plants (black trace line), but not by undamaged AR1-infected plants (pink trace line).

3.5 Discussion

The results from this study provide the first evidence that ASW adults utilise olfaction to orient towards the volatile blend released by perennial ryegrass, demonstrating that ASW are capable of detecting hosts from a distance. This is in contrast to the study of Pilkington (1987) who assessed weevil response to endophyte-free perennial ryegrass (cultivar 'Nui') and identified no chemotactic behaviour. We assume that no responses were observed because a four-arm-olfactometer with dynamic airflow was used and the maximum observation time was one hour per weevil. It is possible that these weevils did not show any chemotactic orientation when exposed to a constant stream of air or maybe the observation time was too short. Hints that ASW could respond to plant volatiles in a still air olfactometer, were derived from unpublished results showing that adult weevils (mixed gender) from an over-wintering (reproductive diapause) generation of ASW respond positively to endophyte-free Italian ryegrass (*Lolium multiflorum*) (J. Dohmen-Vereijssen, personal communication). Host-searching has also been assessed in a congeneric species, *Listronotus maculicollis*, which is a pest of turf in the United States of America. Like ASW, females of this species were shown to move towards the volatile

blend released by their host plant, *Poa annua* (McGraw *et al.*, 2011). *Poa annua* is considered a volunteer grass species in New Zealand and is a known host of ASW along with Italian ryegrass, tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratensis*), maize (*Zea mays*), barley (*Hordeum vulgare*) and wheat (*Triticum* spp.) (Pottinger, 1961; Kain & Barker, 1966; Barker *et al.*, 1983; Jensen *et al.*, 2009). It would be of interest for future studies to identify whether the volatile blends emitted by each of these hosts are attractive to ASW and whether weevils show any preference.

In this study we were unable to demonstrate that plant volatiles are involved in endophyte-mediated defence of perennial ryegrass. It is likely that perception of the endophyte by Argentine stem weevil is mediated by either contact chemoreception or an initial post-ingestional malaise and this will be investigated in a subsequent chapter. Exploiting volatile emissions to avoid endophyte-infected plants can be advantageous for host-searching insects; energy may be conserved and the insect may avoid ingesting harmful metabolites. However, the usefulness of exploiting such cues in New Zealand's intensive pastoral ecosystems is unknown. ASW did not co-evolve with *Epichloë festucae* var. *lolii* and perhaps selection pressure on ASW has not sufficed for them to evolve an ability to identify and avoid the volatile blend emitted by endophyte-infected hosts in New Zealand's pastoral ecosystems. ASW have been in New Zealand for >100 years but it is only in the last 40 years that endophytes have been commercialised and farming has intensified to the point that lowland pastures are regularly renewed. The primary defensive mechanism of these endophytes appears to be bioactive alkaloids which, evidence suggests, have antifeedant rather than strictly toxic effects on adults (Popay *et al.*, 1990). Antifeedants exert less selection pressure than toxins as the insect can find alternative hosts and survives to reproduce whereas ingestion of toxic plant material will result in death. Furthermore, *in planta* alkaloid concentrations are known to fluctuate as they can be strongly influenced by many abiotic and biotic factors (Thom *et al.*, 2013; Hennessy *et al.*, 2016). As a result, it is possible for endophyte-infected plants to contain alkaloid concentrations which are below bioactive thresholds for all or part of the year (Ball *et al.*, 1991; Popay *et al.*, 2003a; Fletcher *et al.*, 2006; Hennessy *et al.*, 2016). The first generation of ASW emerge in the spring when alkaloid concentrations are lower. In addition, farmland in New Zealand contains low species diversity (Goldson *et al.*, 2020) which means that thousands of perennial ryegrass plants are grown in close proximity, allowing weevils to move between plants without expending too much energy.

The greater attraction of weevils to plants damaged by conspecific insects, observed in this study, is likely advantageous for host-searching weevils in New Zealand's pastoral ecosystems. Endophyte-free plants are damaged more frequently and to a greater extent than plants infected with bioactive endophyte strains that produce peramine (Popay & Wyatt, 1995; Popay *et al.*, 1999), a strong deterrent to adult weevils (Rowan *et al.*, 1990), and therefore movement towards HIPVs may assist in locating favourable endophyte-free hosts. This attraction could also be disadvantageous for host-

searching weevils as HIPVs can attract other insects which compete for the same resource. In addition, HIPVs are known to act as indirect plant defence mechanisms, attracting predators and parasitoids of the attacking insect (Suckling *et al.*, 2012; Mutyambai *et al.*, 2015). The parasitic wasp, *Microctonus hyperodae*, was introduced to New Zealand to control ASW and although responses to HIPVs have never been assessed it is conceivable that this wasp could also utilise these chemical cues when searching for weevils to parasitise. The primitive habitat and 'centre-of-origin' of the ASW is thought to be in the 'Mallines' (form of wetland) of Argentina where population sizes are believed to be small (Lloyd, 1966). In these habitats ASW may have relied on HIPVs to locate mates, as plant volatiles can be detected over greater distances than insect pheromones (Dickens *et al.*, 1993; Ruther *et al.*, 2000), as well as aiding in location of dispersed hosts. Locating mates in New Zealand's pastoral ecosystems is unlikely to be as challenging for this weevil as populations as high as 436 individuals per m^2 have been reported (Goldson *et al.*, 1998a, 1999).

The response towards damage was also documented in a previous study where significantly more over-wintering (reproductive diapause) ASW (mixed gender) oriented towards damaged Italian ryegrass plants (endophyte-free, cultivar 'Tama', tetraploid grass) over equivalent undamaged plants (J. Dohmen-Vereijssen, personal communication). Positive responses towards damaged host plants have been observed in other weevil species such as the pepper weevil (Addesso *et al.*, 2011) and the vine weevil (*Otiorhynchus sulcatus*, Coleoptera: Curculionidae), but for the latter this interaction was dependent on the host plant, as damaged yew (*Taxus baccata*) and spindle trees (*Euonymus fortune*) were attractive but *Rhododendron* and strawberry (*Fragaria x ananassa*) were not (Van Tol *et al.*, 2002). Volatile analyses performed in this study found that damaged plants released higher concentrations of several compounds including cis- and trans- β-ocimene and released indole. Indole is a known HIPV and is induced by damage in several other species including maize (*Zea mays*) (Degen *et al.*, 2012; Erb *et al.*, 2015), lima bean (*Phaseolus lunatus*) and cotton (*Gossypium hirsutum*) (McCall *et al.*, 1994). Erb *et al.* (2015) demonstrated that indole may act as an aerial priming agent in maize, preparing systemic tissues and neighbouring plants for insect attack. Indole was emitted by one of the five replicates of undamaged plants assessed in this study. It may be that indole is produced constitutively as variability between individual plants can be high or this may have been a result of damage from another pest, such as an aphid or mealybug, or perhaps accidental mechanical damage.

Nine of the thirteen compounds emitted by perennial ryegrass were able to be tentatively identified in the present study and endophyte was found to have an effect on the quantities and identities of the volatile compounds emitted. These results have demonstrated that endophyte-infected plants do emit unique profiles, thus presenting potential odour cues for insects to exploit. The volatiles emitted by perennial ryegrass have been analysed in two previous studies (Pańka *et al.*, 2013a; Qawasmeh *et al.*, 2015). Pańka *et al.* (2013a) collected and identified the volatiles released by three genotypes of

perennial ryegrass collected from Poland and Austria. Endophyte-free plants and plants infected with an unidentified strain of *Epichloë festucae* var. *lolii* were sampled. Pańka *et al.* (2013a) reported a list of 8 volatiles, three of which, linalool, indole and cis-β-ocimene (also known as (Z)-ocimene) were identified in the current study. Despite assessing the same cultivar ('Grasslands Samson') and endophyte strain (AR1), I did not identify any of the 18 volatile compounds reported by Qawasmeh*, et al.* (2015) in their analysis of endophyte-free and endophyte-infected (AR1, CT and AR37) perennial ryegrass. Although some of this variation may be explained by differences in collection methods as well as host plant genotype, age (12 weeks vs 25 weeks) and environmental conditions, further investigation of the volatiles released by perennial ryegrass is required. In the present study both qualitative and quantitative differences were identified between the volatile blends emitted by endophyte-infected and endophyte-free hosts. Yue *et al.* (2001) also reported both quantitative and qualitative differences in the blend emitted by endophyte-infected (*Epichloë coenophiala*) and endophyte-free tall fescue, but Qawasmeh *et al.* (2015) and Pańka *et al.* (2013a) reported only quantitative differences. The mechanisms underlying endophyte-mediated changes in volatile emissions have yet to be understood (Qawasmeh *et al.*, 2015; Rostás *et al.*, 2015).

Sex-specific responses to host plants have been documented in the literature with females often showing stronger responses to plant volatiles than males (Szendrei & Rodriguez-Saona, 2010; McGraw *et al.*, 2011). For this reason, the current study focused on olfactory responses of female ASW which are responsible for selecting hosts that will provide a suitable resource for larval development. Due to the difficulty involved in sexing live ASW using morphological features alone, a small number of male ASW were accidentally included in this study. A suggestion of an effect of sex on host selection was identified in one olfactometer experiment where a smaller percentage of males selected AR1-infected plants than females. However, little can be extrapolated from this result as the population size was small (19 males). Future studies should look to repeat the experiments and methodology reported here to explore and compare the foraging behaviour of male and female ASW adults.

The parasitic wasp, *M. hyperodae*, was introduced to New Zealand as a biological control agent for ASW in 1991 (Goldson *et al.*, 1993). Although its release was initially successful (Goldson *et al.*, 1994), a decline in parasitism has recently been documented and this has corresponded with reports of an increase in pasture damage (Popay *et al.*, 2011; Goldson *et al.*, 2014; Goldson *et al.*, 2015). To explain this decline, it has been proposed that the weevil has evolved resistance to this parasitoid (Goldson & Tomasetto, 2016; Tomasetto *et al.*, 2018a). In the present study, ASW were collected from the field and behaviour assessed in an olfactometer within 48 hours. Although it is possible to purge parasitoids from a population of field collected weevils, this was not done as parasitism rates were expected to be low and rearing insects in a colony could have influenced their behaviour and selection in olfactometer experiments. Weevils were dissected following the experiment to investigate weevil selection in

response to parasitism. This is because parasitoids have been known to influence the behaviour of their host (Weinersmith, 2019). A significant difference was found in one of the still-air olfactometer experiments, whereby a higher percentage of not parasitized weevils selected CT-infected ryegrass compared to parasitized weevils. This is an interesting finding given that the CT endophyte negatively affects both the weevil and the parasitoid larvae (Barker & Addison, 1997). However, it is important to consider that this analysis was performed on the response of just 32 parasitized weevils. Nevertheless, this is an interesting finding and future studies may look to build on these results and gather further data by repeating the experiments documented here using parasitized weevils.

To conclude, ASW adults were able to utilise olfaction to orient themselves towards the volatiles released by perennial ryegrass and weevils displayed a preference for plants previously damaged by conspecific weevils. However, we found no evidence that ASW use olfaction to distinguish between endophyte-infected (AR1 and CT strains) and favourable endophyte-free plants. We hypothesise that ASW rely on cues gathered after they have contacted the plant to detect and avoid plants that contain bioactive endophyte strains. These results contrast with two previous studies which determined that two beetle species were able to exploit volatile blends to avoid endophyte-infected grasses. Research investigating the mechanisms involved in perception of endophyte by host-searching insects are sparse and further research is required to fully understand these complex interactions as insights may identify opportunities for improving endophyte-mediated control of agricultural grasses.

Chapter 4

Behavioural Responses of Argentine Stem Weevil (*Listronotus bonariensis*) to Endophyte-infected (*Epichloë festucae* var. *lolii*) Perennial Ryegrass (*Lolium perenne*)

4.1 Abstract

Asexual fungal endophytes (*Epichloë* spp.) colonise agricultural grasses (Poaceae) in a defensive mutualistic interaction that provides host plants with protection against phytophagous pest insects. Antifeedant effects have been well documented but the mechanisms involved in perception of endophyte by host-searching insects are not known. Observational studies were designed to evaluate effects of endophyte (common-toxic and AR1) on behaviour (feeding, stationary, walking, grooming and mating) and host-selection of Argentine stem weevil (ASW) adults (*Listronotus bonariensis*). Observations of weevil feeding and position in a no-choice and multiple-choice experiment indicate that ASW are able to perceive endophyte and, as a result, fewer weevils selected endophyte-infected plants for sustained feeding. An equal number of weevils recorded on endophyte-infected and endophyte-free hosts during the first assessment in the choice experiment, suggests that ASW do not utilise pre-contact cues (olfaction or vision) to avoid endophyte. In both choice and no-choice tests, ASW exhibited strong aversion responses to the endophyte. In choice experiments, only eight of 45 weevils tested were observed feeding on AR1-infected plants and only one weevil was observed to have fed on both hosts during the observational period, suggesting that post-ingestional perception is unlikely. In the no-choice experiment grooming of sensory appendages was only observed in weevils enclosed with endophyte-infected (both AR1 and CT) and not endophyte-free plants. The current study provides strong evidence that ASW rely on contact chemoreception to perceive and avoid bioactive endophytes in otherwise acceptable host plants. This study is the first to our knowledge to demonstrate an antagonistic effect of endophytes on insect mating behavior.

4.2 Introduction

Asexual fungal endophytes of the genus *Epichloë* colonise agricultural grasses in a defensive mutualistic interaction (Clay, 1988). Asexual morphs do not have an external form and grow as unbranched hyphae through the intracellular spaces of the host plants cells (Philipson & Christey, 1986). In this relationship the endophyte receives shelter, nutrients and a means of transmission and in return the host gains increased protection from abiotic and biotic stressors (Prestidge *et al.*, 1982; Rowan & Gaynor, 1986; Hennessy *et al.*, 2016; Malinowski & Belesky, 2019). Anti-insect properties of asexual *Epichloë* morphs are well known and the relationship is exploited in pastoral ecosystems to

reduce insect damage and improve herbage production in several countries including New Zealand, Australia and the USA (Johnson *et al.*, 2013; Young *et al.*, 2013; Ferguson *et al.*, 2018). Anti-feedant effects of endophyte are regularly documented in glasshouse and field trials but there is a poor understanding about the effect endophyte has on insect behaviour and in particular, how the behavioural processes leading up to host plant selection and acceptance are affected by endophyte and endophyte-derived alkaloids. A greater understanding of insect behaviour in the presence of endophyte will provide insight into how insects perceive and subsequently reject endophyte-infected plants that would otherwise be acceptable host plants. It has, perhaps, been assumed that perception of endophyte is mediated by ingestion of endophyte-derived alkaloids, resulting in a malaise and an avoidance response. Although a post-ingestional malaise is one theory it is also feasible that insects detect endophyte via sensory perception before ingesting plant material. Sensory perception involves olfactory and/or contact (gustatory) chemoreception and is often referred to as an insect's 'sense of smell and taste'. Hair-like projections, known as chemosensilla, detect odours (olfactory sensilla) and the major nutrients essential for survival, such as amino acids, carbohydrates and secondary plant compounds (gustatory sensilla) from a distance (olfactory) and after contacting the plant surface (olfactory and gustatory) (Bernays & Chapman, 1994; Chapman, 2003; Bruce *et al.*, 2005; Schoonhoven *et al.*, 2005). Mechanisms of perception are explored in this study using Argentine stem weevil adults (ASW, *Listronotus bonariensis* (Kuschel, 1955), Coleoptera: Curculionidae) and endophyte-infected (*Epichloë festucae* variant *lolii* Latch, M. J. Chr. & Samuels, Hypocreales: Clavicipitaceae) perennial ryegrass (*Lolium perenne* Linnaeus, Poales: Poaceae) as a model system.

New Zealand's pastoral-based production is significantly impacted by native and exotic phytophagous insects. In a recent review Ferguson *et al.* (2018) calculated that the mean annual cost of pest damage can reach $2.3 billion NZD in an 'average year' with costs likely to be substantially higher in pest outbreak years or when compounded by extreme climatic events (Ferguson *et al.*, 2018). The Argentine stem weevil is a major pest of perennial ryegrass (Prestidge *et al.*, 1991), the predominant agricultural grass species in New Zealand. Highly mobile adults feed on the leaves and young seedlings before depositing eggs under the outer sheath of the pseudostem. After larvae hatch, they burrow into and mine the centre of the plant before pupating in the soil. An integrated pest management strategy involving endophyte and a parasitic wasp, *Microctonus hyperodae* (Hymenoptera: Braconidae), has been implemented to help control ASW populations in New Zealand's intensive pastoral ecosystems (Goldson *et al.*, 2005; Ferguson *et al.*, 2018).

The 'naturalised' common-toxic (CT) endophyte strain as well as the 'selected' AR1 strain, which was introduced from Europe and commercialised in New Zealand in 2001, provide perennial ryegrass with protection against adult ASW. This has been demonstrated in pot trials and in the field where fewer adult feeding scars are recorded on endophyte-infected plants (Barker *et al.*, 1984b; Popay *et al.*, 1999;

Popay & Thom, 2009). The CT endophyte strain is known to produce the alkaloids lolitrem B, ergovaline and peramine whereas AR1 produces peramine but not lolitrem B and ergovaline (Johnson *et al.*, 2013). In the previous chapter I investigated olfactory responses of female ASW adults to perennial ryegrass in still-air olfactometer experiments. ASW were able to utilise olfaction to locate and orient themselves towards host plants (endophyte-free perennial ryegrass) but weevils were not able to distinguish between endophyte-free and endophyte-infected (CT and AR1) hosts using olfaction alone. Olfactometer results suggest that ASW must contact potential hosts to perceive endophyte.

In this study, choice and no-choice experiments using whole plants were designed to explore behavioural differences relating to host plant selection by ASW adults in response to endophyte. The aims were to: (1) compare weevil behaviour on favourable (endophyte-free) and unfavourable (AR1 and CT endophyte-infected) plants, (2) investigate how ASW select endophyte-free hosts for feeding when presented with a choice and (3) observe and interpret behaviour prior to host-selection. Results from these experiments will lead to a basic understanding of the mechanisms involved in perception of endophyte and will outline areas that require further research.

4.3 Methods

4.3.1 Establishment of ryegrass plants and endophyte testing

Endophyte-infected (AR1 and CT) and endophyte-free perennial ryegrass plants (*Lolium perenne* cultivar 'Grasslands Samson') were established from seed supplied by the Margot Forde Germplasm Centre (Palmerston North, New Zealand). Fifty seeds were placed into a Petri dish (90 mm) lined with damp filter paper (1 mL cold tap water) and left to germinate in a 20°C controlled environment room for 7 - 10 days in the dark. After germination seeds were planted into individual, identifiable positions in seed trays filled with potting mix (Daltons™) where they were watered with tap water and regularly trimmed.

When plants were at least 6 weeks old they were tested for endophyte infection using a tissue print immunoassay protocol. At least one tiller per plant was cut within 5 mm of the base of the plant. Dead sheaths were removed and the cut surface pressed firmly onto a piece on nitrocellulose paper. Blotted sheets were developed by Jan Sprosen (AgResearch) following an immunoblot protocol described by Simpson *et al.* (2012) with minor modifications (detailed in 2.3.1) (Lyn Briggs and Jan Sprosen, AgResearch Ruakura).

Twenty plants of each treatment (endophyte-free, AR1-infected and CT-infected perennial ryegrass) were prepared for the no-choice behavioural study. Individual plants (15 - 16 weeks old) were removed from seed trays and reduced in size by splitting tillers off from the crown of the plant. A single ramet

consisting of approximately 10 tillers was re-potted into a small pot (75 mm in diameter) with fresh potting mix (Daltons™) and left to establish in a screen house (December, summer).

Fifteen replicate AR1 and endophyte-free plants (21 – 22 weeks) were prepared for the choice test by removing tillers from the base to leave 8 tillers per plant. The plants were then re-potted onto either side of a large pot (120 mm in diameter), an equal distance from the rim (30 mm) and left to establish in a screenhouse (January, summer) for at least 10 days before inclusion in an experiment.

4.3.2 Argentine stem weevil (*Listronotus bonariensis*)

ASW adults were collected from a research farm (AgResearch, Ruakura Research Centre, Hamilton, New Zealand) using a reverse modified blower vacuum designed to collect small insects from pasture. Weevils were collected 24 - 72 hours before inclusion in an experiment. Weevils collected more than 24 hours before an experiment were held in unsorted sample containers that included plant debris and other insects. To starve ASW for the no-choice experiment, weevils were sorted into individual 30 mL containers 24 hours before the experiment began. The containers were covered with damp paper towels and held in a container in a controlled environment room (18°C). For the choice experiment, weevils were placed into specimen containers (3 per 75 mL) and held in the laboratory overnight. ASW used in the no-choice experiment were collected between the 11th and 19th December 2017 and ASW used in the choice experiment were collected between the 23rd and 30th of January 2018. Following each experiment ASW were frozen and later dissected to determine their sex and whether each weevil had been parasitized by *M. hyperodae*.

4.3.3 Observational cage

Observational cages were produced using transparent overhead projection (OHP) film (210 x 295 cm). OHP film was rolled into a cylindrical shape and secured firmly over the top of each potted plant using double sided tape. A square of fine mesh fabric was glued (hot melt adhesive/hot glue) to the top of the OHP cylinder to allow air flow. A new cage was produced for each plant in each experiment to prevent contamination.

4.3.4 Weevil labelling

ASW used in the choice experiment were marked to distinguish individual weevils in the experimental arena. I investigated four methods for marking the weevils: fluorescent powder (Radiant Colour Company, Richmond, CA, U.S.A.), marker pen, correction fluid and glueing a piece of coloured paper onto the weevil. Small marks were applied to the elytra to avoid contaminating chemoreceptors that may occur on antennae, mouth parts and tarsi. The marker pen and coloured paper were found to be unsuitable for marking ASW as the pen was not visible over time and the coloured paper often fell off.

A Petri dish (90 mm) bioassay involving cut leaves (endophyte-free perennial ryegrass cv. 'Grasslands Samson') was run to establish whether the fluorescent powder or correction fluid affected ASW feeding. There were no significant ($P > 0.05$) differences in feeding between either treatment or the control (no marking). Fluorescent powder (Figure 4.1) was subsequently chosen for the experiments as it was easy to apply to live weevils, did not have a strong odour and did not affect feeding or survival of weevils.

Figure 4.1: Argentine stem weevil (*Listronotus bonariensis*) adult marked with a small amount of orange fluorescent powder.

4.3.5 No-choice experiment

Immediately before the beginning of the experiment the top layer of potting mix was removed from each pot and a layer of dry vermiculate spread over the surface. On each of four consecutive evenings, five replicate plants of each treatment (endophyte-free, AR1-infected and CT-infected perennial ryegrass) were arranged in a randomised block design (shown in Figure 4.2) and five ASW adults were enclosed on each plant (4 weevils were added to replicate 20). Weevils were added to each plant 1.5 hours before the first assessment to allow for a settlement period. Assessments of position and behaviour of the weevil were made hourly from 4 pm with a total of four assessments completed for each replicate plant. Assessments were conducted in the evening to optimise feeding behaviour. Positions recorded were: leaf, pseudostem, ligule, crown, dead material, surface, caged area (included pot, OHP and roof) and young tiller. Behaviours recorded were: feeding (head and rostrum (snout) in contact with plant material and moving, feeding scar may or may not be visible), moving (included walking and moving around on the spot), stationary (with normal posture), crouching, mating, grooming (of tarsi or antennae) and oviposition. This experiment was carried out in December 2017 (summer) under natural lighting conditions and at an average temperature of 25°C. The number of feeding scars and eggs present on each plant were counted after the experiment.

Figure 4.2: Argentine stem weevil (*Listronotus bonariensis*) adults enclosed in an arena containing an endophyte-free, AR1-infected or common toxic-infected perennial ryegrass plant (*Lolium perenne*). Red dotted line represents the plants in one block.

4.3.6 Choice experiment

ASW adults were enclosed in a larger (120 mm in diameter) arena with an endophyte-free and AR1-infected plant (Figure 4.3). To prevent weevils from burrowing into the soil a piece of fine mesh was placed over the surface of the potting mix and secured to the base of the plant using a small piece of fine wire. To enable ASW adults to be distinguished from one another in each arena a small amount of fluorescent powder (pink, orange and an uncoloured weevil) was applied to the elytra using a fine tipped paint brush. Three starved weevils were introduced to each arena, in the central position between the two plants. For the first 1 h 45 min in replicates 1 – 3 and 1 h 30 min in the remaining replicates, assessments of position and behaviour were made approximately every 15 minutes (assessment 1 in replicates 1 – 3 was made after 5 minutes from the introduction of the weevils into the arena). The remaining assessments were made every 30 minutes from then on, as weevils began to settle. Five replicates were set up on each of 3 days between 24th January and 1st February 2018 (summer) to give a total of 15 replicates. Between 15 and 17 assessments were made for each weevil. Positions and behaviours were identical to those listed for the no-choice experiment with the exception that surface was not distinguished from the rest of the caged area (pot/OHP/mesh roof) and crouching behaviour was combined with stationary behaviour. The experiment was carried out between 8:30 am and 4 pm at an average temperature of 26°C.

Figure 4.3: Argentine stem weevil (*Listronotus bonariensis*) adults enclosed in an arena with an endophyte-free and AR1-infected perennial ryegrass plant (*Lolium perenne*).

4.3.7 Statistical analyses

No-choice experiment

All analyses were carried out using Genstat 18th edition and graphs were created in Excel. An ANOVA was performed on the mean percentage (average over the four assessments divided by the total number of weevils added times one hundred) of weevils found out of the total number of weevils released. In analysing the data in this way, I assume that the weevils that were not found were not missed from one of the positions recorded but rather they were buried (or partially buried) in the vermiculite. Any errors were anticipated to be random and there was no systematic bias between treatments. An ANOVA was run with the blocking structure of day plus replicate within day. Contrasts were included in the analyses to compare behaviours on endophyte-free to that on endophyte-infected plants. Significance was determined using Fisher's Unprotected least significant difference post hoc test conducted at the 5% confidence level. An ANOVA (blocking structure of day plus replicate within day) was also performed on mean percentage (average over the four assessments divided by the total number of weevils added times one hundred) data to investigate interactions between behaviour (grooming, mating, walking and stationary) and position (on or off (caged arena) the plant). Significance was determined using Fisher's Unprotected least significant difference post hoc test conducted at the 5% confidence level.

An unbalanced ANOVA was performed on the total number of feeding scars (leaf + pseudostem + young tiller damage) found on each plant with the blocking structure of day plus replicate within day. Significance was determined using Fisher's Unprotected least significant difference post hoc test conducted at the 5% confidence level. Three replicates were removed from this analysis as not all

weevils were removed directly following the experiment and thus could have caused more feeding damage to these plants subsequent to the experimental period.

Choice experiment

The percentage (total observations in a position divided by the number of assessments times one hundred) of weevils in each position in the arena (AR1-infected plant, endophyte-free plant, caged arena) was analysed with an ANOVA blocked by replicate. Significant differences were determined using Fisher's Unprotected least significant difference post hoc test conducted at the 5% confidence level.

4.4 Results

4.4.1 No-choice experiment

The aim of this experiment was to observe the effects of endophyte on ASW adult behaviour. Five weevils were enclosed on each plant but not all were found at each of the four assessment points. Weevils not found were likely buried in the vermiculite which covered the surface of the potted plant. The most commonly observed behaviour in all three treatments was stationary followed by walking (Table 4.1). There were more observations of weevils on the cage (includes OHP case, mesh roof and rim of pot) when enclosed with an endophyte-infected plant and fewer weevils were observed at the crown of the plant when compared to endophyte-free plants.

Table 4.1: Total number of Argentine stem weevil adults (*Listronotus bonariensis*) observed in each position and performing each behaviour in the no-choice whole plant (*Lolium perenne*) experiment. Values in parentheses represent the percentage of weevils out of the total found. Values in parentheses in 'total found' column represent the percentage found out of the total number of observations that could have been made if all weevil were visible at each observation. Treatments: Perennial ryegrass infected with the AR1 endophyte strain (AR1), common-toxic endophyte strain (CT) or endophyte-free (EF). Pseudo = pseudostem. Dead m. = dead material. Young t. = young tiller.

	Behaviours						
	Stationary	Moving	Feeding	Mating	Grooming	Crouching	Total found
AR1	102 (43%)	84 (36%)	14 (6%)	26 (11%)	8 (3%)	2 (1%)	236 (60%)
CT	113 (50%)	77 (34%)	14 (6%)	14 (6%)	7 (3%)	2 (1%)	227 (57%)
EF	99 (42%)	64 (27%)	36 (15%)	38 (16%)	0 (0%)	1 (0%)	238 (60%)

	Positions							
	Surface	Cage	Leaf	Pseudo	Crown	Dead m.	Young t.	Ligule
AR1	51 (22%)	47 (20%)	36 (15%)	32 (14%)	27 (11%)	21 (9%)	13 (6%)	8 (4%)
CT	45 (20%)	45 (20%)	52 (23%)	36 (16%)	24 (11%)	13 (6%)	5 (2%)	8 (3%)
EF	51 (21%)	12 (5%)	40 (17%)	42 (18%)	50 (21%)	11 (5%)	13 (5%)	20 (8%)

Significant differences in behaviour and position were found between treatments. On average significantly more weevils were observed feeding on endophyte-free plants than endophyte-infected (AR1 and CT) plants ($P = 0.003$, r.d.f. = 38, s.e.d = 1.713) (Figure 4.4). Fifteen observations of self-grooming were recorded, but none of these were by weevils enclosed with an endophyte-free plant ($P = 0.007$ AR1 vs EF, $P = 0.017$ CT vs EF, r.d.f. = 38, s.e.d = 0.699). Mating behaviour was higher on endophyte-free plants when compared to CT-infected plants ($P = 0.061$ CT vs EF, $P = 0.305$ AR1 vs EF, r.d.f. = 38, s.e.d = 3.37). Interestingly, there were no significant ($P > 0.05$) differences in moving or stationary behaviour between treatments. No observations of oviposition were made and crouching behaviour was rarely observed. There was no significant difference in the total number of weevils found in each treatment ($P = 0.885$, r.d.f. = 38, s.e.d = 6.24).

The mean percentage of weevils was significantly higher on the crown of endophyte-free plants ($P = 0.043$ AR1 vs EF, $P = 0.023$ CT vs EF, r.d.f. = 38, s.e.d = 2.99) and significantly lower ($P = 0.007$ AR1 vs EF, $P = 0.011$ CT vs EF, r.d.f. = 38, s.e.d = 3.07) on the cage (rim of pot, OHP case, roof, does not include the surface). There was also a significant difference in the mean percentage of weevils observed on the ligule with a significantly higher percentage on endophyte-free plants ($P = 0.05$ AR1 vs EF, $P = 0.05$ CT vs EF, r.d.f = 38, s.e.d = 1.511). There were no significant ($P > 0.05$) differences between treatments in the number of weevils observed on the leaves, pseudostems, dead material, young tillers or on the vermiculite surface.

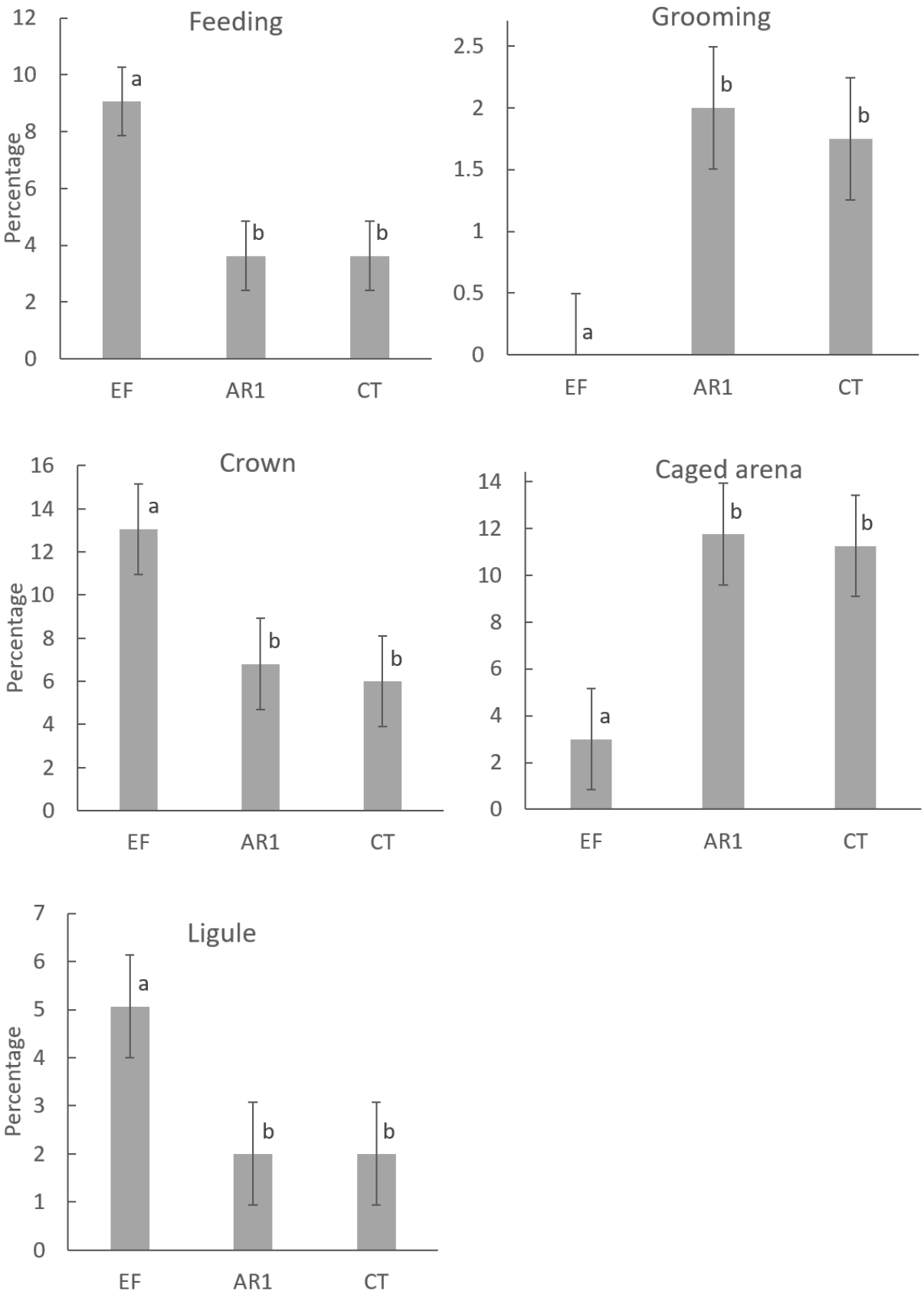


Figure 4.4: Mean percentage found out of the total number of weevils released in each replicate arena in the no-choice behavioural experiment. Graphs represent only the behaviours (Graphs 1 - 2) and positions (Graphs 3 - 5) which were significantly ($P < 0.05$) different between treatments:

endophyte-free (EF), AR1-infected (AR1) and CT-infected (CT) perennial ryegrass (*Lolium perenne*). Caged arena refers to ASW found on the rim of the pot, OHP case and the roof (vermiculite surface is a separate category). +/- standard error of the mean. Letters denote significant differences between treatments; analysis of variance, P < 0.05.

When analysing mating behaviour there was a significant (P < 0.05) treatment by position interaction between weevils enclosed with a CT-infected plant and an endophyte-free plant (Table 4.2). The mean percentage of weevils observed mating was higher on endophyte-free plants than on CT-infected plants but when looking at mating behaviours off the plants (i.e rim of pot, OHP case, roof and vermiculite surface) there were no differences between treatments. There were no significant interactions between treatment and position for grooming, stationary or moving behaviour.

Table 4.2: Mean percentage of Argentine stem weevil adults (*Listronotus bonariensis*) observed performing each behaviour on and off (i.e OHP case, pot and vermiculite surface) the plant in the no-choice behavioural experiment. P-values and l.s.d are presented for each level of the interaction. Different letters above bars indicate significant differences; analysis of variance, P < 0.05.

Endophyte	Position	Mating ($\bar{x}$)	Grooming ($\bar{x}$)	Stationary ($\bar{x}$)	Moving ($\bar{x}$)
EF	On	7.5	0	19.3	8.81
	Off	2.5	0	5.8	7.25
AR1	On	2.75	0.5	19.4	8.5
	Off	3.5	1.5	7.1	12.5
CT	On	1	0.5	21.1	8.31
	Off	2.5	1.25	7.9	11.06
P $_{\text{Treatment}}$		0.12	**0.032**	0.735	0.393
P $_{\text{AR1 vs EF}}$		0.234	**0.016**	0.788	0.181
P $_{\text{CT vs EF}}$		**0.041**	**0.034**	0.441	0.368
P $_{\text{Position}}$		0.475	0.082	**<0.001**	0.251
P $_{\text{Treatment x Position}}$		0.081	0.445	0.968	0.286
P $_{\text{AR1 vs EF x Position}}$		0.07	0.222	0.807	0.132
P $_{\text{CT vs EF x Position}}$		**0.041**	0.359	0.951	0.242
l.s.d $_{\text{Treatment}}$		3.11	0.808	5.05	3.638
l.s.d $_{\text{Position}}$		2.539	0.66	4.13	2.97
l.s.d $_{\text{Treatment x Position}}$		4.398	1.143	7.15	5.145

Damage assessment

The total number of feeding scars (leaves + pseudostems + young tiller) was significantly (P = 0.013, average s.e.d. = 2.79, r.d.f. = 35) higher on endophyte-free perennial ryegrass plants than on plants

infected with the AR1 or CT endophyte (Table 4.3). The difference between AR1 and CT was not significant. Feeding scars were primarily found on the adaxial side of the leaves but several scars were also found on the pseudostem of endophyte-free plants. Although the number of young tillers was greater on CT (n = 80) and AR1-infected (n = 63) plants than on endophyte-free (n = 31), more feeding scars were found on the young tillers of endophyte-free plants. In total, 23 eggs were found on the pseudostems and 13 of these were on endophyte-free plants.

Table 4.3: Sum of the feeding scars and eggs laid on AR1-infected (AR1, n = 20), common-toxic (CT, n = 19) and endophyte-free (EF, n = 18) perennial ryegrass (*Lolium perenne*) plants by Argentine stem weevil adults (*Listronotus bonariensis*) in the no-choice behavioural experiment.

Treatment	**Feeding scars**				**Eggs laid**
	Leaf	Pseudostem	Young tiller	Total no.	Total no.
EF	173	55	23	251	13
AR1	99	5	12	116	7
CT	125	0	12	137	3

Weevil dissections

A sub-sample of 53 weevils from the second week of the experiment (replicates 11 - 20) were frozen and later dissected to determine the sex ratio and parasitism levels in this experiment. The sex ratio was 25 females: 28 males and only 1 parasitized weevil was found.

4.4.2 Choice experiment

The aim of this experiment was to investigate host-selection behaviour of ASW when weevils are presented with a choice between an endophyte-free and AR1-infected perennial ryegrass plant. On average, weevils were observed on one of the plants (average 83%) more often than they were observed on the cage arena (surface, pot, OHP case, roof). Of this, significantly more ($P < 0.004$, s.e.d. = 8.56, r.d.f. = 74) weevils were observed on endophyte-free plants (average 54%) than AR1-infected plants (29%).

The most commonly observed behaviours were stationary (26%) and feeding (18%) on endophyte-free as well as stationary on AR1-infected plants (16%) (Figure 4.5). Mating was observed between two individuals on an endophyte-free plant and two individuals on an AR1-infected plant. Mating was not observed off the plant in the caged arena. The mating event on the endophyte-free plant was observed over four consecutive observations, indicating that the mating event lasted for at least one and a half hours. These weevils may have continued to mate but the experiment came to an end. The mating

event on the AR1-infected plant was observed over three consecutive observations, equating to at least one hour. Oviposition was not observed at any point during this experiment. Weevils were observed grooming (23 observations in total) on both AR1-infected (7 observations) and endophyte-free plants (8 observations) as well as the caged arena (8 observations) and both male (11 observations, 5 weevils) and female (12 observations, 8 weevils) weevils displayed this behaviour.

The most commonly observed position was on the leaves of endophyte-free plants (31% of observations) (Figure 4.6). Interestingly, there were a similar number of observations on the crown of endophyte-free and AR1-infected plants (1.9% and 1.3%, respectively).

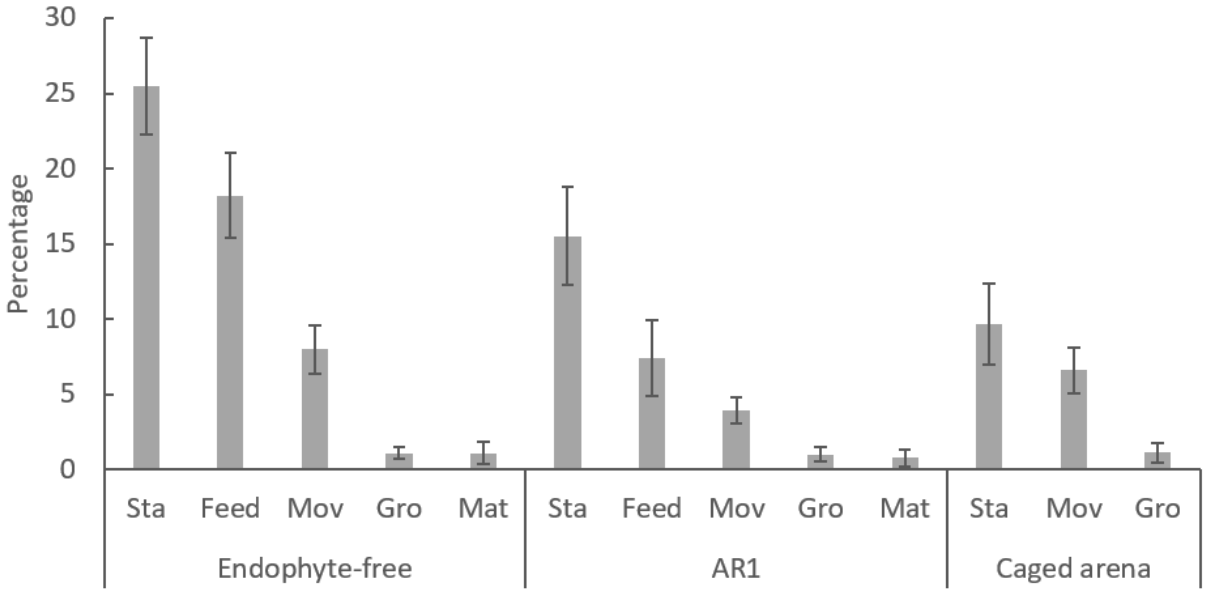


Figure 4.5: Behaviours displayed by Argentine stem weevils adults (*Listronotus bonariensis*) on endophyte-free and AR1-infected ryegrass (*Lolium perenne*) as well as the caged arena (OHP case plus the surface and roof) in the choice experiment. Behaviours recorded were stationary (Sta), feeding (Feed), moving (Mov), grooming (Gro), mating (Mat). +/- standard error.

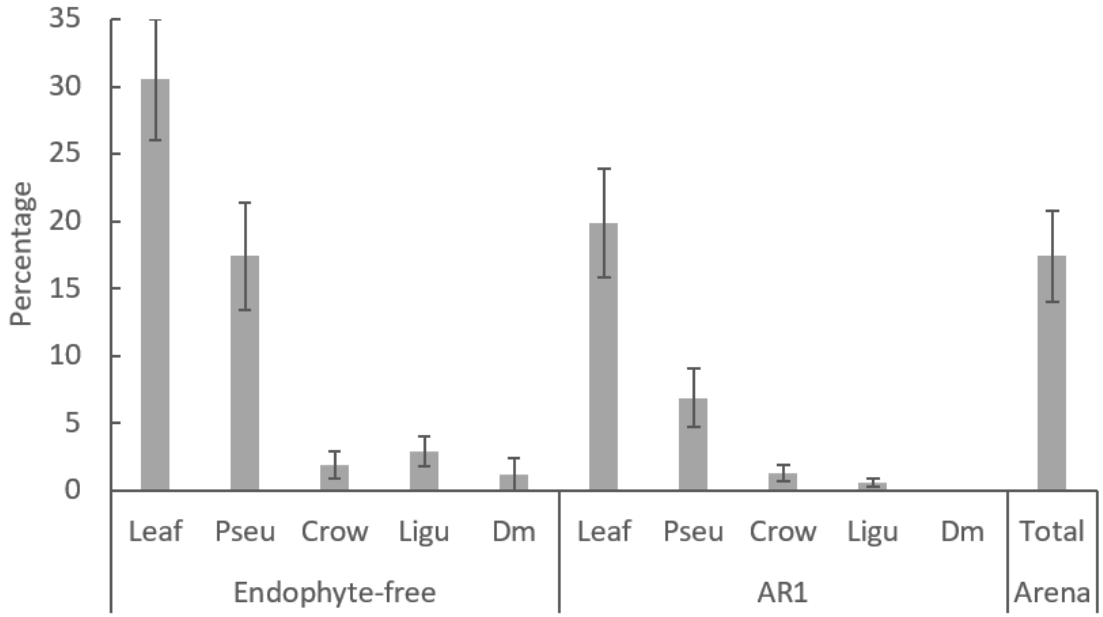


Figure 4.6: Position of Argentine stem weevil adults (*Listronotus bonariensis*) on the caged arena (OHP case plus surface and roof), endophyte-free and AR1-infected ryegrass (*Lolium perenne*) in the choice experiment. Positions recorded were leaf, pseudostem (Pseu), crown (Crow), ligule (Ligu) and dead material (Dm). +/- standard error.

Host-selection

At the first observation (15 minutes) 11 weevils were found on an endophyte-free plant and 11 weevils were found on an AR1-infected plant (12 remained in the caged arena, 2 were not found; R1 - 3 were not included as the first observation occurred at 5 minutes) (Figure 4.7). Which plant a weevil was first observed feeding on was also documented. Thirty-two weevils were first observed feeding on endophyte-free plants compared to 9 on AR1-infected plants (4 were not observed feeding).

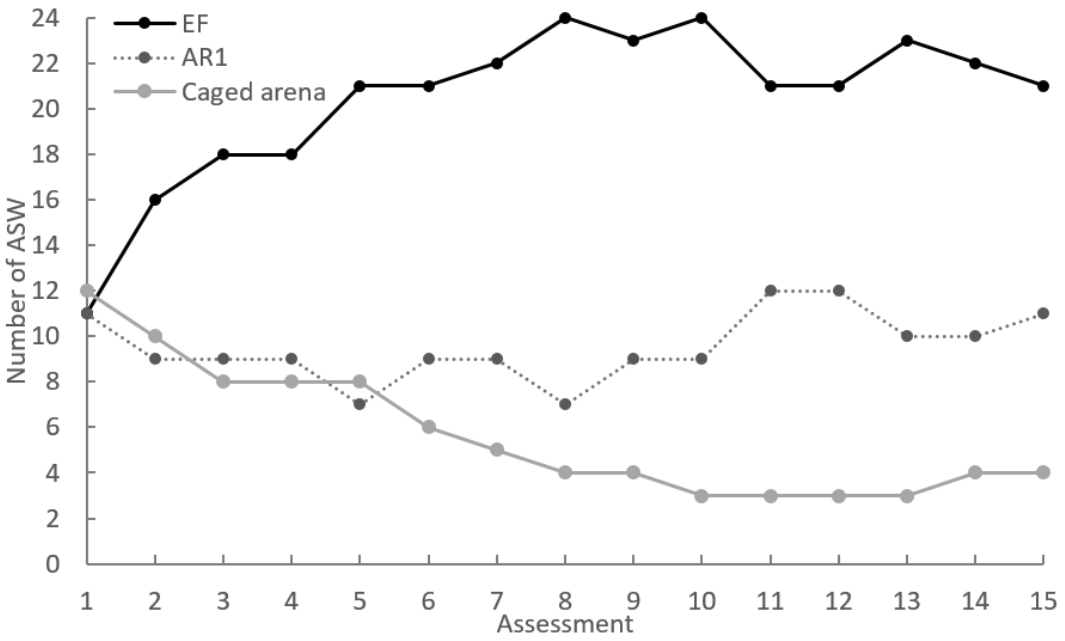


Figure 4.7: Number of Argentine stem weevil (ASW, *Listronotus bonariensis*) observed on each plant (*Lolium perenne*) at each assessment in the choice experiment. Assessments were every 15 minutes for the first 5 assessments and every 30 minutes thereafter (these data do not include replicates 1 – 3).

In this experiment I have defined host-selection as the plant a weevil selected to feed on over at least two observational periods to take into account possible 'test biting' behaviour. Feeding observations may be sequential or involve a break, but during the break the weevil must not have been observed off the plant or on the alternative host plant. Of the 45 weevils that were observed in this experiment, 25 selected endophyte-free plants for 'sustained feeding', 8 selected AR1-infected plants and 12 did not select a host plant for feeding (Figure 4.8, note: Figure 4.8 does not display replicates 1 – 3).

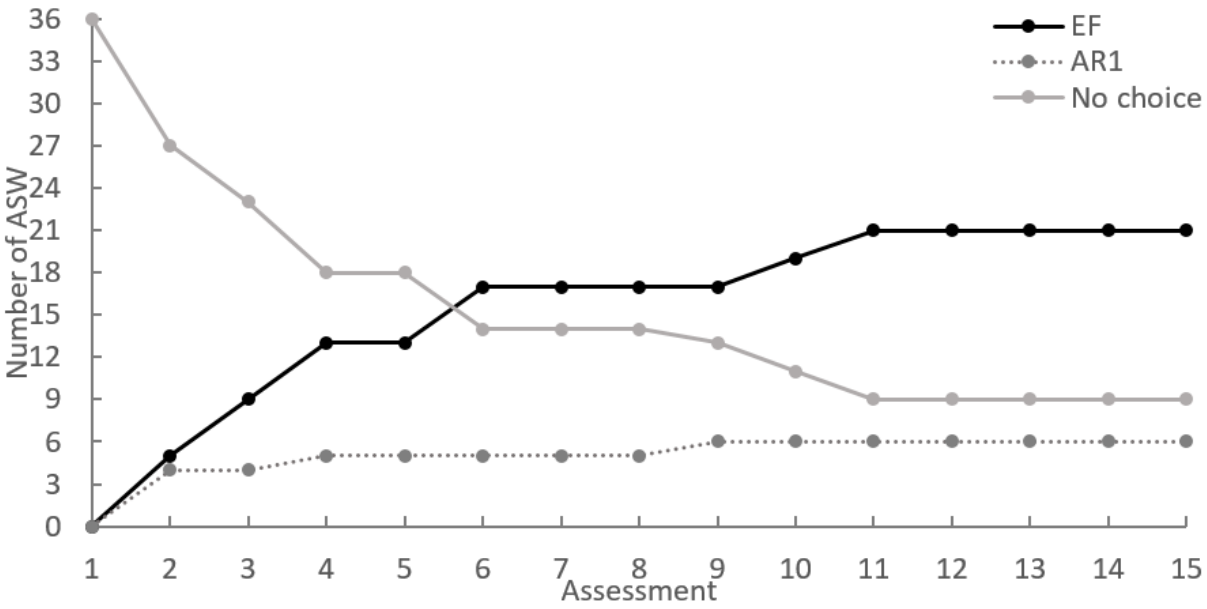


Figure 4.8: Cumulative number of Argentine stem weevil (ASW, *Listronotus bonariensis*) that had accepted a host plant (*Lolium perenne*) for sustained feeding (observed feeding on a plant over at least two observation periods) at each assessment in the choice experiment. No choice represents the number of weevils that had not selected a host plant for sustained feeding at each assessment point. Assessments 1 - 5 are every 15 minutes and every 30 minutes thereafter (these data do not include replicates 1 – 3).

Effect of sex on host plant selection

At the end of the observational period weevils were frozen and later dissected to explore effects of sex on host plant selection. The sex ratio was 23 females: 22 males. A similar number of males and females selected each host plant (Figure 4.9).

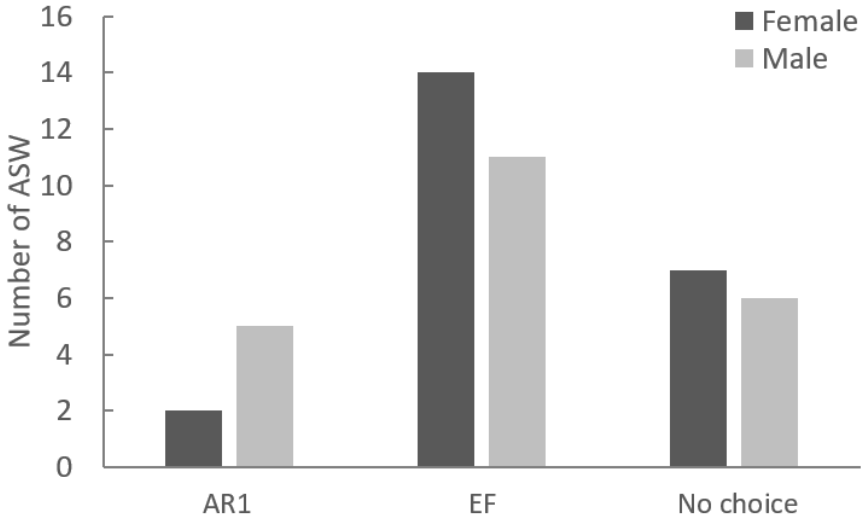


Figure 4.9: Total number of Argentine stem weevil (ASW, *Listronotus bonariensis*) that selected the endophyte-free (EF) or AR1-infected (AR1) perennial ryegrass (*Lolium perenne*) plant for feeding in the choice experiment.

Effect of parasitism on host plant selection

Of the 45 weevils involved in this study just two were confirmed to contain a parasitoid larva. Both weevils selected a host-plant for feeding. The female selected the endophyte-free plant and the male selected the AR1-infected plant.

4.5 Discussion

Choice and no-choice whole plant experiments were carried out to explore behavioural differences relating to host plant selection by ASW adults in response to endophyte. The majority of ASW selected endophyte-free plants for sustained feeding and egg laying and were thus able to perceive endophyte. Weevils did not take long to respond to endophyte as the number of weevils observed on AR1-infected plants in the choice experiment started to decrease at the second observation. Exactly how they were able to perceive endophyte cannot be conclusively determined from these behavioural assessments alone, but results highlight a number of promising areas for future research.

It has been well documented that both the AR1 and CT endophyte strains have an anti-feedant effect on ASW adults. This has been demonstrated in pot trials and in the field where fewer feeding scars are found on endophyte-infected plants (Barker *et al.*, 1984b; Popay & Wyatt, 1995). Feeding trials, however, cannot be used to infer perception mechanisms as they do not provide sufficient information to distinguish between sensory perception and deterrence as a result of ingesting a toxic compound.

Sensory perception involves olfactory and/or gustatory chemoreception. In the previous chapter I investigated whether ASW adults utilise olfaction to detect endophyte (AR1 and CT) before contact is made with the plant. Although endophyte was found to alter the volatile blend released by perennial ryegrass, ASW were not able to exploit those differences to select their favoured endophyte-free host plants. Observations from the whole plant choice experiment performed here support this result as a similar number of weevils were found on endophyte-free and endophyte-infected (AR1) plants at the first assessment (11 each, not including R1 - 3). If ASW utilised olfaction I would have anticipated that more weevils would have oriented towards endophyte-free plants from the outset.

Contact (gustatory) chemoreceptors are found on mouthparts, tarsi and antennae and enable insects to detect chemicals in solution as well as on the dry surface of the leaf (Roessingh *et al.*, 1991; Chapman, 2003; Popescu *et al.*, 2013). Before selecting a host for feeding, insects may explore the surface of the plant, performing behaviours such as tarsal drumming or scratching, palpation and antennation (Chapman & Sword, 1993; Headrick *et al.*, 1996). These behaviours bring contact chemoreceptors on appendages into contact with the plant material, allowing the insect to gather information about the suitability of the plant as a host. Observations of palpation and antennation behaviour were not recorded in this study as weevils are small (3.3 – 4.4 mm) and hold their head close to the plant surface making it difficult to accurately assess these behaviours in real time. However, antennal movements were seen in this study and further studies should explore this behaviour as contact with the surface would indicate a contact chemoreceptive function, whereas antennal 'waving' may point to an olfactory function. Two of the most frequently observed behaviours in this study were of weevils remaining stationary or moving (walking and moving around on the spot) on plant material (as opposed to the caged arena/surface), providing evidence that tarsi were in direct contact with the surface for long periods of time. Pilkington (1987) noted sensilla on ASW tarsi and proposed that the features of some sensilla, such as the presence of a distal pore, suggested a uniporous contact chemoreceptive function of individual sensilla. However, this is speculative and further investigation through transmission electron microscopy and detailed electrophysical studies are required to establish their true function. If receptors are confirmed to occur on tarsi, simply standing on the leaf could provide ASW with information about the suitability of the plant as a host for feeding (Chapman & Bernays, 1989).

Deterrent effects of both the CT and AR1 strains have previously been linked to endophyte-derived alkaloids and it is probable that host-selection is governed by the presence of these compounds. Peramine (both strains) and ergovaline (CT only) were shown to have an anti-feedant effect on ASW adults when these compounds were incorporated into a semi-synthetic insect diet (Popay *et al.*, 1990; Rowan *et al.*, 1990). No significant difference in weevil behaviour or position were observed between weevils caged on CT and AR1 infected plants, suggesting that peramine is the major driver for the

deterrent response observed here. It appears that peramine is essential for perception of endophyte as ASW adults were also not deterred from feeding on ryegrass infected with the AR37 endophyte (Popay & Wyatt, 1995), which does not produce peramine, or an AR1-infected plant lacking a gene (*per*A) essential for peramine biosynthesis (Tanaka *et al.*, 2005).

It has yet to be determined whether peramine can be found on the dry surface of the leaf. However, this compound is known to occur in the guttation fluid of endophyte-infected perennial ryegrass (Koulman *et al.*, 2007) and thus ASW may detect peramine via direct contact with the guttation fluid or perhaps contact after the fluid has dried on the leaf surface. Peramine concentrations on the surface of infected plants could be evaluated in the future by rubbing the surface of the leaf onto a piece of solvent soaked filter paper and analysing the extract by liquid chromatography – mass spectrometry.

A role of contact chemoreception in perception of endophyte is further supported by observations of grooming behaviour. In the no-choice experiment, only weevils caged with an endophyte-infected plant (AR1 and CT) were observed grooming. This behaviour was characterised by self-grooming of tarsi and antennae by rubbing tarsi together and rubbing tarsi over antennae. It is intuitive that sensory appendages should be cleaned or groomed regularly to maintain sensitivity to environmental stimuli. Böröczky *et al.* (2013) analysed groomed and ungroomed antennae of the American cockroach (*Periplaneta americana*, Blattodea: Blattidae) and through emission gun scanning electron microscopy and gas chromatography identified an accumulation of cuticular hydrocarbons (CHCs) on the antennal surface. This substance was also found on chemosensilla and covered the pores that odorants and non-volatile compounds must move through when entering the sensillum. Grooming behaviour removed excess CHCs from the surface and electroantennography confirmed that groomed antennae were significantly more responsive to a sex pheromone, geranyl acetate and hexanol than ungroomed antennae (Böröczky *et al.*, 2013). Observations of the carpenter ant (*Camponotus pennsylvanicus*, Hymenoptera: Formicidae) and housefly (*Musca domestica*, Diptera: Muscidae) also found a build-up of CHCs on ungroomed antennae, suggesting that grooming performs this function in a range of insect taxa that employ different grooming methods (Böröczky *et al.*, 2013). The CHC layer can absorb compounds from the environment and, if this layer is not cleaned, compounds may stimulate receptor neurons after the source of the stimulus has moved (Böröczky *et al.*, 2013). Endophyte-infected plants are known to contain lipophilic compounds, so it is possible that they are present on the plant surface and absorb into the CHC layer, triggering cleaning behaviour to maintain high temporal acuity of sensory organs. Alternatively, chemoreception of a deterrent alkaloid may trigger this behaviour directly. Detection of nicotine hydrogen tartrate, a deterrent compound, by free-walking *Schistocerca americana* (Orthoptera: Acrididae) was shown to trigger 'leg-raising' behaviour in this insect to avoid tarsal contact with the surface (White & Chapman, 1990).

The next stage in an insect's evaluation of a potential food source and host-plant may involve damaging the plant to release plant fluids. Insects may achieve this by taking a small 'test bite', macerating or probing (as is the case for sucking insects) plant material. This behaviour provides the insect with additional sensory information as it exposes contact chemoreceptors that are located around the mouthparts to compounds that may be present in plant fluids. This could also be an important aspect of ASW chemoreception as peramine has been found in the cut leaf fluid of endophyte-infected ryegrass (Koulman *et al.*, 2007). 'Test bites' were not directly observed in the behavioural experiments presented in this study, but Barker *et al.* (1984b) reported that ASW 'sampled' endophyte-infected leaves in a Petri dish choice test, although no data were presented to support this observation. Pilkington (1987) also observed ASW taking 'test bites' on the leaves of endophyte-free and CT-infected plants in a Petri dish arena (four weevils observed over two hours). Whether the 'test bites' noted by these authors involved ingestion is not clear. Therefore, I established a Petri dish comb test to observe 'test biting' behaviour of ASW adults. A single weevil was enclosed in a Petri dish with an endophyte-free and an AR1-infected leaf. Behaviour was monitored continuously for up to 3 hours and 12 replicates were completed. 'Test bites' were not observed and only two weevils selected a leaf for feeding. ASW spent most of their time moving, stationary or grooming themselves in the Petri dish. Given the lack of host-acceptance observed using this method I suggest a clip cage design, using the leaves of live plants for further investigation of 'test biting' behaviour.

A post-ingestive mechanism would indicate that ASW are not able to perceive endophyte using sensory stimuli before ingesting plant material. This can be hazardous for host-searching insects as ingestion of toxins can have adverse effects on fitness. An example from the literature of a rapid post-ingestional feedback are larvae (5^{th} instar) of the tobacco hornworm (*Menduca sexta*, Lepidoptera: Sphingidae) that were previously naive to nicotine. Larvae fed on a diet containing nicotine for no longer than 30 seconds before they suddenly stopped feeding, often displaying toxic responses such as twitching and writhing. Results suggested a post-ingestional feedback rather than a contact chemoreceptive response because ablation of mouthpart receptors did not alter the response and sensory recordings did not suggest a role of chemosensory sensilla (Glendinning, 1996). Root aphid, *Aploneura lentisci*, feeding on ryegrass infected with the AR37 endophyte strain are also affected by a post-ingestive toxin. In this case aphids did not appear to detect endophyte using olfactory or contact chemoreception as equal numbers were found on endophyte-infected and endophyte-free plants (chapter 2). Popay and Cox (2016) noted that, after 7 – 20 days, root aphid that establish on endophyte-infected plants succumb to a toxin. Tremors suggest the involvement of a neurotoxin and the delayed response suggest a slow acting toxin or an inducible secondary compound. It appeared as though aphids were unable to terminate feeding, often displaying symptoms of toxicity while the stylet was still inserted in

the root. Further investigation is required to establish whether ingestion is fatal or whether aphids can recover and go on to search for an alternative host-plant.

When presented with a choice in the present study ASW tended to select endophyte-free plants for sustained feeding indicating that ASW adults, unlike root aphid, can initially perceive endophyte. In the choice experiment, 91% of the weevils were recorded as feeding in at least one assessment during the observational period. Of this just 17.1% were observed to have fed on AR1-infected plants and only 2.4% fed on both hosts during the observational period. If a post-ingestional effect was involved I would have expected to observe a higher number of weevils feeding on AR1-infected plants before moving to feed on favourable endophyte-free plants. However, behaviour was not monitored continuously and it is possible that some instances of feeding may have been missed.

The olfactory and behavioural results presented in this chapter and that of my previous study (chapter 3) suggest that ASW adults detect endophyte (CT and AR1) through contact chemoreception. Future studies should look to build on these results by identifying the location of chemosensilla on ASW sensory appendages using transmission electron microscopy as this will allow ablation experiments to be carried out. In ablation studies, sensory appendages that contain contact chemosensilla, such as tarsi, antennae or palps may be excised and further behavioural tests carried out. When combined with results from the present behavioural analyses, results from these studies would provide a stronger understanding of contact chemosensory deterrence as it relates to this interaction.

In addition to selecting host-plants for feeding, females must also select suitable hosts for oviposition. It has been demonstrated in this and previous studies (Barker *et al.*, 1984b; Popay *et al.*, 1995; Popay & Wyatt, 1995) that female ASW lay fewer eggs on endophyte-infected (AR1 and CT) ryegrass plants. Although it is reasonable to suggest that oviposition deterrence is linked to feeding deterrence it is also possible that a separate mechanism is involved. Oviposition was one of the behavioural categories in this study but was not observed in either experiment although eggs were laid. This is not surprising as weevils were not continuously observed and oviposition could be a rapid process. Pilkington (1987) reported that the process took less than 5 minutes, although this was based on the observation of only one weevil. Future experiments may look to film the sequence of behaviours leading up to oviposition to gain a basic understanding of this process and to determine at what stage of the hierarchical testing sequence of behaviours is disrupted by endophyte.

Ovipositional deterrence is a likely explanation for the reduced number of eggs on endophyte-infected plants in this study. Results from this study, also show that endophyte can influence other aspects of the reproductive process. For weevils found on the plant (rather than the caged arena) in the no-choice experiment the mean percentage of weevils observed mating on endophyte-free plants was higher than CT-infected plants, suggesting that endophyte affects mating behaviour. I am not aware of

another study to report a negative association between *Epichloë* endophytes and the reproductive behaviour of ASW, or any other insect species, and thus this finding appears to be novel. It is perhaps not surprising to observe effects of endophyte on ASW mating as the reproductive behaviour of herbivorous insects is often integrated with host plants. Host-plant chemistry is known to impact sexual communication for some insect species, either by influencing the production and release of pheromone signals or the detection response to those pheromones (Hughes & Renwick, 1977; Deng *et al.*, 2004; Schmidt-Büsser *et al.*, 2009; Xu & Turlings, 2018). It is possible that perception of endophyte disrupts pheromone signalling, culminating in a lower incidence of mating behaviour on infected plants. More work is necessary to confirm this finding and in doing so it would be interesting to investigate success of mating events, gender-specific responses as well as further investigation of effects on duration of mating events. The two mating events documented in the choice experiment indicated that mating may be longer on endophyte-free plants, but further investigation is required. It is important to determine whether there is a separate mechanism involved in ovipositional deterrence as it is vital that these mechanisms are conserved when selecting new endophyte strains for pest management.

This study appears to be the first to report an effect of *Epichloë festucae* var. *lolii* on both the grooming and mating behaviours of an herbivorous pest insect. In addition to contributing towards a greater understanding of the interaction detailed in this study, this information can be used to inform relationships between ASW and other species. An example is the multitrophic interaction between ryegrass, endophyte, ASW and its parasitoid, *Microcotonus hyperodae*. A study conducted by Goldson *et al.* (2015) reported that endophyte (AR1, CT and AR37) had no effect on ASW parasitism rates in the field. This is surprising given that ASW are more frequently found and spend more time feeding on endophyte-free plants. When displaying feeding behaviour the caudal end of the weevil is exposed, making the weevil more susceptible to parasitism (Phillips, 2002). Given this information it could have been argued that higher parasitism rates should have been found in endophyte-free plots. However, grooming behaviour also exposes the weevil to parasitism (Phillips, 2002) and thus the observation of increased grooming on endophyte-infected plants provides a possible explanation as to why no significant differences were observed in the field.

In conclusion, endophyte was shown to have a significant effect on the behaviour of ASW adults when weevils were enclosed with a single plant and when they were presented with a choice. ASW can perceive endophyte and the evidence collected thus far indicates that contact chemoreception is the primary mechanism involved. Further studies are required to confirm this interesting new finding.

Chapter 5

General Discussion

Despite 40 years of research into *Epichloë* fungal endophytes of grasses there is still much we do not know. A major gap in our knowledge is an understanding of how phytophagous pest insects perceive and subsequently avoid plants infected with bioactive endophyte strains. I investigated whether two pasture pest species were able to initially perceive endophyte and then investigated both pre- and post-contact mechanisms that may be involved in perception.

5.1 Perception of endophyte by Argentine stem weevil (*Listronotus bonariensis*)

Still-air olfactometer experiments conducted in this thesis have demonstrated that ASW adults utilise olfaction to aid in location of perennial ryegrass host plants. ASW were shown to select perennial ryegrass over a control and weevils preferred damaged over undamaged ryegrass, demonstrating an ability to distinguish and select between volatile blends. However, ASW were unable to distinguish between endophyte-infected and endophyte-free ryegrass using volatile blends.

The olfactory and behavioral studies reported in this thesis suggested a role of contact chemoreception (gustation) in perception of endophyte by ASW adults. Four lines of evidence were presented that supported this theory. In the olfactometer bioassays; (1) there was no evidence that ASW avoided the odour blend released by endophyte-infected perennial ryegrass before (AR1 or CT) or after (AR1) plants had been damaged by conspecific insects. In the whole plant choice experiment; (2) there was no evidence that ASW utilised precontact cues (olfaction and vision) to orient away from endophyte-infected (AR1) plants from the outset; (3) ASW showed a strong aversion to endophyte-infected plants with just eight of 45 weevils observed feeding on AR1-infected plants and only one weevil feeding on both hosts during the observational period, suggesting a post-ingestional mechanism is unlikely. In comparison, 32 weevils were observed feeding on endophyte-free plants (4 did not feed). In the whole plant no-choice experiment; (4) Grooming of sensory appendages was observed in weevils enclosed with endophyte-infected plants (both AR1 and CT), but not endophyte-free plants. Thus, it was concluded that contact chemoreception is the primary mechanism that ASW adults use to perceive the AR1 and CT endophyte strains in perennial ryegrass, but further investigation is required to confirm this interesting new finding.

Older pastures in New Zealand, especially those in hilly country, are likely to be infected with the CT endophyte strain while newer stands are typically planted with ryegrass containing one of the 'selected' endophyte strains, AR1 or AR37. Here I investigated behavioural and olfactory interactions

with the AR1 and CT strain as fewer ASW adult feeding scars have been observed on these plants in pot trials and in the field (Barker *et al.*, 1984b; Popay & Wyatt, 1995; Popay *et al.*, 1999). Although results inform the interaction between weevils and these particular strains, it seems likely that ASW would interact in a similar way with strains that have a similar chemical profile (i.e. produce peramine). Further investigation is required to understand the diversity in perception mechanisms that may exist among different strains of *E. festucae* var. *lolli*. It would be of interest for future studies to investigate whether strains such as AR37, which is not considered to be active against the adult life stage, but is active against larvae (Popay & Wyatt, 1995), alters behaviours associated with host-searching, selection and acceptance.

This was the first study to explore the mechanisms of perception employed by ASW adults. Experiments were designed to observe effects of endophyte on different aspects of behaviour and results have highlighted the most promising areas for future research to build on. Future studies should identify the location of chemosensilla on ASW using transmission electron microscopy. This would allow ablation and electroantennography experiments to be carried out. Electroantennography may be carried out to investigate whether known contact chemosensilla respond to whole plant extracts or specific endophyte-derived alkaloids (Schiestl & Marion-Poll, 2002; Fraser *et al.*, 2003; Balakrishnan *et al.*, 2017). This technique is useful for investigating sensory responses as it provides information on olfactory perception, but it does not provide information about the behavioural response elicited (Schoonhoven *et al.*, 2005). Ablation experiments typically involve surgical removal of appendages that contain contact chemosensilla, blocking sensilla using a wax or deactivation using an acid (Rajashekar & Shivanandappa, 2017; Simmonds *et al.*, 2019). Further evidence for sensory deterrence could be provided if the deterrent response was altered following ablation. However, caution should be applied when interpreting results from these experiments as interfering with appendages may alter the movement and behaviour of some insects. When combined with results from behavioural analyses, results from electrophysical and ablation experiments would provide a stronger understanding of contact chemosensory deterrence as it relates to these interactions.

When considering evidence from previous semi-synthetic diet experiments (Rowan & Gaynor, 1986; Popay *et al.*, 1990; Rowan *et al.*, 1990) it seems likely that perception of the AR1 and CT endophyte strains is mediated by detection of the endophyte-derived alkaloids, peramine and possibly ergovaline (CT strain only), by chemosensilla. However, it is also possible that chemosensory deterrence is mediated by another unknown compound. Further investigation using the techniques described above will help to elucidate this interaction.

In addition to identifying mechanisms involved in perception, these experiments identified an effect of endophyte on mating and grooming behaviour of ASW adults. I am not aware of any other study to

report negative effects of an asexual *Epichloë* endophyte strain on these behaviours in ASW, or any other pasture pest, and thus these findings are novel. It is not surprising that these findings do not appear to have been reported previously as studies investigating anti-insect properties of *Epichloë* endophytes typically report outcomes, such as the number of feeding scars or eggs, over a given period of time, rather than investigating the behavioural processes which may be responsible for these outcomes (Prestidge *et al.*, 1982; Barker *et al.*, 1983, 1984a; Barker *et al.*, 1984b; Clay & Cheplick, 1989; Popay & Wyatt, 1995; Popay & Baltus, 2001; Popay *et al.*, 2005; Timper *et al.*, 2005; Jensen *et al.*, 2009; Hennessy *et al.*, 2016; Ruppert *et al.*, 2017; Shymanovich & Faeth, 2018). Therefore, this research highlights the need to increase the number of behavioural studies in the endophyte literature as important effects are most likely being missed.

Research carried out in the early 1980s established a link between the naturalized CT endophyte and resistance to ASW in New Zealand (Prestidge *et al.*, 1982). It was subsequently established in semi-synthetic diet bioassays that peramine, an alkaloid derived from this strain, deterred ASW adults from feeding (Rowan & Gaynor, 1986; Popay *et al.*, 1990; Rowan *et al.*, 1990). This knowledge was used to screen hundreds of endophyte strains collected from Europe, with the aim of identifying a strain that produced peramine but not the mycotoxins associated with livestock toxicosis (Tapper & Latch, 1999). This led to the discovery and subsequent commercialization of AR1. In New Zealand, the uptake of perennial ryegrass infected with the AR1 endophyte strain was rapid and 1570 tonnes of infected seed was sold in the first two years after commercialization (Milne, 2007). At the time, this represented half of the perennial ryegrass market and this value likely increased over the next few years, until the release of AR37 in 2007 (Milne, 2007). Although the mechanism of perception utilised by insects was not understood, the behavioural response displayed by ASW was able to be exploited to select the AR1 endophyte strain for commercialization. In the present study, olfactometer bioassays have demonstrated that ASW adults are able to utilise olfaction to orient towards perennial ryegrass. This represents a natural behavioural response which could be exploited to improve control and increase pastoral production in the future. Studies could utilise the volatile blend documented in this study to identify the compounds essential for ASW attraction. The attractive volatile blend could then be used to develop novel crop management strategies such as trap cropping and lures. Gene editing techniques may also be utilised in the future to alter plant volatile blends so that ASW are unable to 'recognise' their hosts prior to physical contact with the plant. Endophyte was found to alter the plant volatile blend in this study and thus it may also be possible to identify an endophyte strain that alters the volatile blend to such an extent that the blend is no longer attractive to host-searching insects.

The results from this research are not restricted to understanding perception methods to inform endophyte research. They can also be used to explain interactions with higher trophic levels, such as the interaction between ASW and its parasitoid *Microctonus hyperodae*. The current study and

previous trials have established that ASW feed more on endophyte-free plants and weevils are more frequently observed on these plants (Barker *et al.*, 1984b; Popay *et al.*, 1995; Gerard, 2000). When feeding, the caudal end of the weevil is exposed and as a result, weevils performing this behaviour are more susceptible to being parasitized by *M. hyperodae* (Phillips, 2002). To investigate effects of endophyte on parasitism rates in the field, Goldson *et al.* (2015) collected and dissected ASW from plots containing endophyte-free and endophyte-infected (AR1, CT and AR37) ryegrass (*Lolium perenne* and *Lolium multiflorum*). Given the information documented above, it may have been surmised that the parasitism rate among weevils collected from endophyte-free plots would be higher than those collected from areas containing endophyte-infected (AR1 and CT) ryegrass, but endophyte was not found to have a significant effect on parasitism. The present study has found that ASW enclosed on endophyte-infected (AR1 and CT) ryegrass increase grooming behaviour. Like feeding, self-grooming exposes the weevil to parasitism (Phillips, 2002). Thus, results presented here may help to explain results from the previous field trial as weevils enclosed on endophyte-free and endophyte-infected plants may both display behaviours that expose them to parasitism.

In response to insect attack plants release herbivore induced plant volatiles (HIPVs) which are known to attract natural predators or parasitoids of attacking insects (Turlings *et al.*, 1990; De Moraes *et al.*, 1998). Olfactory responses of *M. hyperodae* to different host weevils and undamaged *Lolium mulitflorum* have been assessed in Y-tube olfactometer experiments in Canada (Cournoyer & Boivin, 2004), but research is yet to address whether this parasitoid is capable of responding to plant HIPVs. In the present study ASW caused less damage to endophyte-infected (AR1 and CT) plants and the volatile blend emitted by damaged plants was altered by endophyte-infection. This raises the question of whether a mutualistic fungal endophyte can alter the searching ability or behaviour of a parasitic wasp.

5.2 Perception of endophyte by *Aploneura lentisci*

Very little is known about the root aphid, *Aploneura lentisci*, in New Zealand and significant questions about their behaviour and life cycle remain unanswered. In this study multiple olfactometer bioassays and a host preference bioassay were carried out to explore host-searching, selection and acceptance behaviour in order to understand the mechanisms involved in perception of a bioactive endophyte strain (AR37). In olfactometer experiments *A. lentisci* were unable to utilise olfaction to orient towards either the roots (two experiments) or herbage (one experiment) of their perennial ryegrass (endophyte-free) host plants. This indicates that under the experimental conditions I used these aphids do not use olfaction to locate hosts and therefore, no further experimentation involving endophyte was carried out. Despite this result, I cannot rule out the involvement of olfactory stimuli as host-searching behaviour can be complex and involve multiple complementary stimuli.

In a host preference assay an equal number of *A. lentisci* initially (24 h) selected endophyte-free and endophyte-infected (AR37) host plants. Results suggested that *A. lentisci* are unable to initially perceive this endophyte using sensory cues or through an initial post-ingestional effect. When the results presented here are combined with those presented by Popay and Cox (2016), it appears as though *A. lentisci* cannot initially perceive and subsequently avoid host plants infected with a bioactive endophyte (AR37). Instead aphids likely succumb to a toxin, possibly a fast-acting neurotoxin or inducible secondary compound, after 7 to 20 days of exposure (Popay & Cox, 2016).

Although transitory, the common-toxic endophyte can have some effect on *A. lentisci* population sizes, but the 'selected' AR1 endophyte strain has no negative effect (Popay *et al.*, 2004; Hume *et al.*, 2007; Popay & Gerard, 2007; Popay & Thom, 2009). AR37 on the other hand is known to provide perennial ryegrass with resistance to *A. lentisci* (Pennell et al., 2005; Popay & Gerard, 2007; Popay & Cox, 2016) and thus experiments focused on perception of this strain. This research has shown that negative effects are not always associated with an initial perception and subsequent avoidance of endophyte-infected host plants. Future studies should work to ascertain the identity of the toxin that affects *A. lentisci* using fractionation techniques and semi-synthetic diet bioassays. Identifying this toxin will allow new endophyte strains to be screened faster in the future as researchers will have an indication of whether a strain of interest is likely to be toxic to this pest before insect screening trials are carried out. It may also be possible to isolate this toxin and utilise it in other pest management strategies.

Aploneura lentisci has only recently been considered a major pasture pest in New Zealand, largely because its habitat is subterrestrial and negative effects are often attributed to other abiotic or biotic factors such as drought. This research has contributed to the small body of literature by identifying that neonate root aphid do not appear to utilise olfaction in host-searching and further elucidating interactions with the AR37 endophyte. Experiments focused on apterous morphs of *A. lentisci* as alatae aphids have only been documented in the literature on two occasions and it is unclear whether these alatae were virginoparae or sexupare (Lowe, 1968; Müller, 2019). Alatae morphs have also never been observed when sampling below ground populations in the field and this led Popay and Cox (2016) to propose that apterous nymphs are primarily responsible for dispersal of the clone in New Zealand. It is possible, however, that alatae virginoparae are produced at certain times of the year, or in response to certain climatic conditions and are involved in dispersal of the clone. To follow on from the findings of this thesis it would be interesting to establish whether alatae aphids are involved in dispersal in New Zealand and if so whether these morphs are able to utilise olfaction to orient towards host plants.

There is a wealth of literature investigating host-selection and acceptance behaviour of above ground aphid species (Pickett *et al.*, 1992; Webster, 2012; Döring, 2014), but despite extensive searching I have not found another study that has investigated olfactory responses of an aphid morph that

inhabits subterrestrial ecosystems. The methodology developed in this study could be built on in future studies to assess responses of other economically important root aphid species such as the lettuce root aphid (*Pemphigus bursarius*, Hemiptera: Aphididae) or other small subterrestrial insects, such as first instar larvae of the African black beetle (*Heteronychus arator*, Coleoptera: Scarabaeidae) or grass grub (*Costelytra giveni*, Coleoptera: Scarabaeidae).

5.3 Mechanisms of perception of endophyte by phytophagous pests in New Zealand's intensive pastoral ecosystems

New Zealand's diverse range of native and introduced pasture pests likely employ a range of mechanisms for perceiving bioactive endophytes. The present study has identified that ASW adults likely utilise contact chemoreception to perceive the AR1 and CT strains while *A. lentisci* appear to be unable to initially perceive the AR37 endophyte strain. In contrast, African black beetle adults were shown to be more attracted to endophyte-free ryegrass than ryegrass infected with the CT or AR1 endophyte strains in Y-tube olfactometer bioassays (Qawasmeh *et al.*, 2015) suggesting possible olfactory perception of these strains. Studies are yet to investigate whether other sensory or post-ingestional mechanisms are involved in mediating perception of these strains. Interestingly, beetles could not differentiate between the odours released by endophyte-free ryegrass and ryegrass infected with the AR37 endophyte strain, which is also active against this species (Ball *et al.*, 1994), suggesting perception of AR37 is mediated by another mechanism such as gustation or a post-ingestional feedback. Larvae of the native New Zealand grass grub feed on the roots of perennial ryegrass plants and the predominant endophyte strains, AR37 and AR1, as well as the naturalized CT strain are unable to provide host plants with protection. Olfactometer bioassays have been carried out to investigate olfactory responses of grass grub larvae to a meadow fescue hybrid grass (*Festuca pratensis* x *Lolium perenne* cultivar GrubOUT®) infected with the endophyte *Epichloë uncinata* (strain U2) (Rostás *et al.*, 2015). Hybrid grasses infected with this endophyte strain can contain lolines in the roots and this compound is thought to be active against grass grub (Popay *et al.*, 2003b; Patchett *et al.*, 2011; Barker *et al.*, 2015). More larvae moved towards endophyte-free grass in a below ground olfactometer, suggesting olfactory perception of this endophyte. I attempted to investigate this interaction further in this thesis but grass grub response rates in the four-arm olfactometer were so low that no conclusive results were obtained.

Intensive pastoral ecosystems in New Zealand are different from the native grasslands of Europe as they are characterised by their lack of species diversity and a community of plants and insects which originate from different areas of the world. The predominant pasture species is perennial ryegrass with swards often planted in mixture with white clover (Charlton & Stewart, 1999), although it is not uncommon for pastures to contain 'weed' grasses such as *Poa annua*, which does not contain endophyte and is a known host of pests such as Argentine stem weevil. When new pastures are sown

in New Zealand it is rare for farmers to select endophyte-free ryegrass as these plants are highly susceptible to insect attack. Instead, newer pastures are commonly planted with seed infected with the AR1 or AR37 endophyte strains although, for reasons explained earlier, swards will contain a mixture of endophyte-infected and endophyte-free plants. There are several highly destructive herbivorous insects in New Zealand that can cause severe damage to both the above and below ground plant parts. Some of these species, such as grass grub and porina, are native and have flourished with the introduction of perennial ryegrass from Europe. Others such as the Argentine stem weevil and African black beetle were accidentally introduced and some of these species are not considered a pest in their home range as population sizes are restricted. There are few natural predators to control insect populations in New Zealand and this has facilitated large population sizes. When investigating host-selection behaviour it is interesting to consider which mechanism of endophyte perception would provide the highest level of plant protection in these ecosystems.

A strong mechanism in pastoral ecosystems may involve an endophyte that insects cannot detect using sensory perception but is toxic once a small amount of plant material is ingested. This would be advantageous as the insect is killed rather than deterred, thus providing protection to neighbouring endophyte-free plants. However, such a high degree of control may result in a species developing resistance. The lack of complexity and co-evolution in New Zealand's pastoral ecosystems makes this a possible outcome. Furthermore, an example of rapid evolution has recently been documented in these ecosystems. The parasitic wasp, *Microctonus hyperodae*, was introduced to New Zealand from South America in 1992 to control ASW populations (Goldson *et al.*, 1993). In the years following its release ASW parasitism rates were found to reach 90% (Goldson *et al.*, 1998b). However, rates have dropped by as much as 50% and this has corresponded with reports of increasing pasture damage (Popay *et al.*, 2011; Goldson *et al.*, 2017). This reduction has led researchers to theorize that sexually reproducing ASW adults have evolved resistance to this parthenogenetic wasp in what is described as an 'unequal evolutionary arms race' (Goldson *et al.*, 2015; Tomasetto *et al.*, 2018a; Tomasetto *et al.*, 2018b).

I hypothesise that robust protection would be conferred by an endophyte strain that is perceptible by an insect at multiple points in the host-searching and acceptance processes. That is, an endophyte that produces a deterrent odour, as well as a compound deterrent to 'taste' sensilla and a compound that deters an insect post-ingestionally. A combined sensory and initial post-ingestional malaise would provide the strongest level of protection. In whole plant experiments performed here, a few ASW selected AR1 for sustained feeding, indicating that they were not deterred from these plants, possibly because they were able to overcome initial sensory deterrence. Similar observations have been observed in pot trials, particularly when insects are confined to endophyte-infected plants and their choice is restricted. It can also occur in the field, where feeding damage is recorded on plants that

contain endophyte (AR1 or CT) (Barker *et al.*, 1984b; Popay & Wyatt, 1995). Concentrations of deterrent compounds are known to vary between host plant species, genotype and between seasons (Ball *et al.*, 1991; Breen, 1992; Ball *et al.*, 1993; Salminen *et al.*, 2005; Pańka *et al.*, 2013a; Hennessy *et al.*, 2016). This variability may alter whether an insect perceives the endophyte and thus whether a deterrent behavioral response is initiated. An endophyte that contains multiple mechanisms of perception that rely on different compounds would strengthen the control provided by endophyte.

Recent studies by Qawasmeh *et al.* (2015) and Rostás *et al.* (2015) have highlighted the potential role that plant volatiles may play in endophyte-mediated defense of their host plant. I identified differences in the plant volatile blend released by endophyte-infected (AR1) and endophyte-free perennial ryegrass in this study but results from insect trials do not support a role of plant volatiles in defense against the above and below ground pests studied here. Plant volatiles are an interesting area for future study and research should focus on identifying which species may utilise these chemical cues as well as investigating the relevance of these compounds in the field where endophyte-infected and endophyte-free plants are grown in close proximity and are often intertwined.

Olfactometers are an essential tool for investigating an insect's sense of smell, but it should be emphasized that developing an olfactometer can be very challenging. This is because devices must be developed to suit the morphological and behavioural features of each test insect and experiments require high response rates. The development stage can take several weeks or months which can be difficult if you are working with a species, such as ASW, which is seasonal. When assessing olfactory responses for an insect which has never been assessed before researchers must consider whether to use a Y-tube design or a multi-arm olfactometer and whether airflow is required. Researchers must also consider the habitat of the species; a below ground root aphid for example will require a substrate such as vermiculite, perlite, sterilized soil or glass beads to simulate the conditions below ground. When developing a protocol for olfactory experiments it is important to consider factors such as temperature, humidity, lighting conditions, duration of the experiment, amount of plant material, number and gender of test insects as well as the time of day or night that their behaviour is usually expressed. During this thesis I attempted to build on a previous study by Rostás *et al.* (2015) to further explore olfactory and gustatory responses of the New Zealand grass grub. Despite successive attempts using the olfactometer design outlined in this paper, grass grub response rates were low. Larvae appeared to move up the arms of the below ground olfactometer but returned to the center after being unable to access plant material. Larvae sitting in the center had a large volume of vermiculite weighing down on them whereas the vermiculite in the arms was easily disturbed. The arms of the olfactometer were also very narrow and grass grub larvae are known to attack each other when enclosed in a small space. I suggest that future studies should either run one larva at a time or look to modify this olfactometer design. Modifications may include creating a 'holding area' at the end of each

arm, greatly increasing the diameter of each arm or adding multiple small chambers to the sides of each arm to act as a refuge for individual grubs.

In conclusion, this thesis explored effects of endophyte on behaviour of two host searching pest insects in order to understand how insects perceive and subsequently avoid hosts that are colonized by mutualistic fungal endophytes. Results have helped to fill a major gap in the literature and have laid down a framework for future research. A greater understanding will ensure that these mechanisms are conserved when selecting new endophyte strains for commercialization in the future. Results from this study are not limited to endophyte research and have broader applications for the development of novel crop management strategies and understanding interactions with higher trophic levels. They are also not limited to pastoral ecosystems in New Zealand as the 'selected' endophyte strains AR1 and AR37 are exported to Australia and parts of North and South America, where these pest insects, or related species, are also found.

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